

**SIGNALING FROM DEPOLARIZATION TO ALTERNATIVE
SPLICING: IDENTIFICATION OF MOLECULAR LINKS
MEDIATING INDUCIBLE EXON SKIPPING**

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Alternative pre-mRNA splicing is one of main contributors to protein diversity. A fundamental unanswered question is how splicing decision is altered by signal transduction. The C1 exon of the glutamate NMDA receptor NR1 subunit is alternatively spliced in a tissue-specific manner. It is predominantly included in the forebrain, and predominantly skipped in the hindbrain. Importantly, the C1 exon encodes the C1 protein cassette containing an ER retention signal that regulates the surface delivery rate of assembled receptors, and a calmodulin binding site that plays critical roles in learning and memory via regulating Ca^{2+} flux. Recently, it has been reported C1 exon skipping can be induced by cell depolarization. However, the molecular links mediating the depolarization on the cell membrane and alternative splicing in the nucleus largely remain unclear. Here I have investigated the roles of Ca^{2+} signaling, PKA and hnRNP A1 in mediating depolarization induced C1 splicing silencing. Specifically, using Ca^{2+} imaging, I have found the KCl induced sustained Ca^{2+} influx is associated with C1 splicing silencing. In addition, I have demonstrated that CaMK IV and PKA regulate C1 splicing in a hnRNP A1 dependent manner. Finally, I have shown that hnRNP A1 is recruited to the C1 splicing site upon depolarization in neurons. The results here have demonstrated signal transduction from depolarization to splicing factors, showing how splicing decision can be altered by external stimuli.

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PREFACE

It has been the most fulfilling five years of my life since I started my Ph.D training in University of Pittsburgh with all the expectations of a new beginning along with a bit of fear from having to stand on my own in a completely different place. This dissertation is an important witness of my experiences in academia, overcoming the difficulties to reach goals that I did not always know I could achieve. I would not be able to make it without the people who walked me through all the good and bad times. Therefore, I would like to take this opportunity to express my deep thankfulness to them.

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With sincere respect and appreciation,

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1.0 INTRODUCTION

Maturation of many eukaryotic pre-mRNAs involves the removal of intervening intron sequences and joining of exon sequences to generate a mature mRNA transcript. The process of splicing involves a two-step chemical mechanism and step-wise assembly of the spliceosome, the macromolecular machinery that catalyzes the splicing reaction. Splicing is a precisely controlled process but requires a great amount of inherent flexibility. An alternative spliced exon can be either included or skipped in the final mRNA. The process is regulated by cis-elements in the pre-mRNA as well as trans-factors. Splicing factors regulate alternative splicing in a tissue- and developmental- specific manner. More recently, it has been shown that splicing patterns are also regulated by external stimuli, which not only increases the diversity of proteins but also provides a good system to study the signal transduction of alternative splicing.

1.1 RNA SPLICING AND SPLICEOSOME

In most eukaryotic genes, the coding regions of genes (exon) are separated by non-coding DNA (intron) which is not involved in protein expression. After transcription, those non-coding intron need to be removed and the exons need to be joined, termed splicing. This process is essential

since most introns do not contain any protein-coding information, and the presence of introns could result in frame shifts.

Although the size of intron varies, the sequences which are essential for the removal of intron are usually close to the immediate vicinity of the exon/intron borders (Stephens and Schneider 1992) (Black 2003). In higher eukaryotes, the 5' splice site is characterized by the consensus sequence AG|GURAGU (where "|" denotes the exon/intron boundary, R=purine, Y=pyrimidine, N=any nucleotide). The consensus sequence for 3' splice site is Y_nNYAG|G (Y_n is referred to as the polypyrimidine-tract). Another important sequence is called the branch site located 18 - 40 bases upstream of the acceptor site. The consensus sequence is YNYURAC, where A is the branch formation site (Figure 1A) (Lamond 1995) (Burge 1999).

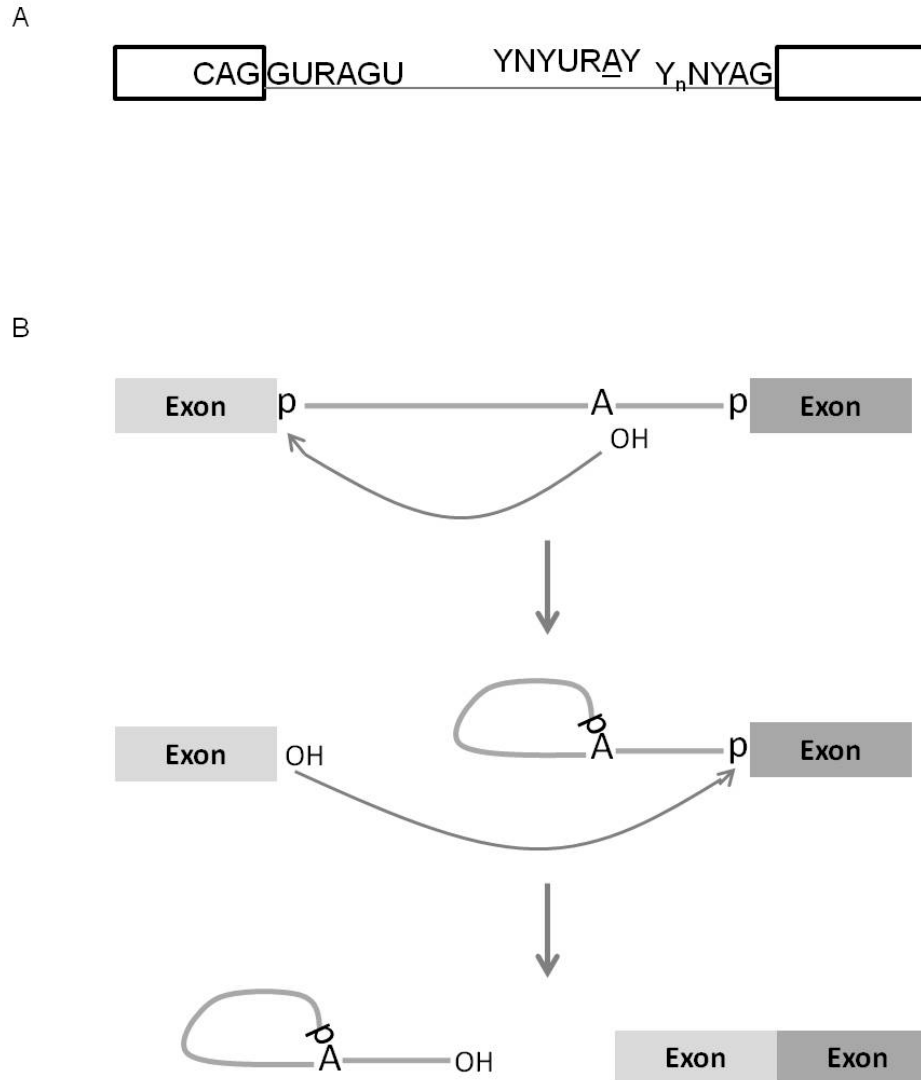


Figure 1. Schematic structure of splicing consensus sequences and two-step chemical mechanism of pre-mRNA splicing.

(A) Consensus sequences of the splicing signals (Lamond 1995). Exon sequences are shown as boxes. Intron sequences are shown in line. A branch site is underlined in bold. A, adenosine; C, cytidine; G, guanosine; U, uridine; R, purine; Y, pyrimidine. (B) A pre-mRNA containing two exons (boxes) flanking an intron sequence (line) with the positions of the 5' splice site (ss), 3' ss, and branchpoint (BP) adenosine (A) is shown (top). The phosphate (p) and hydroxyl (OH) groups involved in catalysis of splicing are also indicated. For splicing, two consecutive chemical transesterification reactions occur resulting in the formation of the spliced exons and intron lariat products.

The splicing of pre-mRNA to mRNA occurs in two steps, both of which involve single transesterification reaction (Figure 1B) (Padgett, Konarska et al. 1984; Ruskin, Krainer et al. 1984). The splicing is initiated by the nucleophilic attack of the 2' hydroxyl group of the branch site adenosine (YNYURAC) on the 3',5'-phosphodiester bond at the 5' splice site, generating a 2'-5' bond at the branch site upstream of the 3' splice site and a free 3' hydroxyl group on the 5' exon. In the second step, the free 3' hydroxyl group on the 5' attacks the phosphodiester bond at the 3' splice site, resulting in joining of the two exons and intron lariat products (Figure 1B) (Grabowski, Zaug et al. 1981; Padgett, Konarska et al. 1984; Konarska, Grabowski et al. 1985; Ruskin and Green 1985). This transesterification reaction is evolutionarily conserved across the self-splicing group II introns of Tetrahymena and the spliceosome-regulated introns of yeast and metazoan pre-mRNA. Similarly, self-splicing group I introns follow a similar chemical mechanism (Grabowski, Zaug et al. 1981). The difference is that guanosine is used as the branch site nucleophile rather than adenosine. Taken together, the chemical mechanism of splicing is highly conserved.

Pre-mRNA splicing takes place within a large highly dynamic complex, termed the spliceosome. It contains core Sm proteins, small nuclear ribonucleoprotein particles (snRNPs U1, U2, U4, U5 and U6) and hundreds of accessory factors (Sharp 1987; Luhrmann, Kastner et al. 1990; Reed 1996). The assembly of a spliceosome on the pre-mRNA is a stepwise pathway and requires the recognition of 5' splice site, 3' splice site and branch site (Reed 1996). Four spliceosomal complexes (complex E, A, B and C) have been identified, which are assembled in an order of E, A, B and C. Before the formation of the spliceosome, the pre-mRNA and hnRNP proteins form a complex in a non-specific manner termed H complex, which is not considered to

be part of the functional spliceosome (Michaud and Reed 1991). The earliest specific event during the formation of spliceosome is the binding of U1 to the 5' splice site, termed E complex. The formation of E complex is independent of ATP. The 5' splice site is defined by the nucleotide base pairs between the 5' end of U1 snRNA and the pre-mRNA (Zhuang and Weiner 1986). The 3' splice site is defined by the U2 snRNP auxiliary factors (U2AF) (Reed 1990). The formation of E complex is a commitment step of splicing reactions, which is highly regulated. Following the binding of U1 snRNP, U2 snRNP binds to the branch site to form pre-splicing complex A. The formation of A complex and subsequent steps are ATP dependent. The U2 snRNP base pairs with the branch site region to form a bulged duplex, with the branch site adenosine unpaired, which is used for the nucleophilic attack in the first transesterification reaction (Query, Moore et al. 1994). In the next step, U4/U5/U6 tri-snRNP is incorporated into the spliceosome in an ATP dependent manner, resulting in the formation of splicing complex B (Bindereif and Green 1987; Konarska and Sharp 1987; Jamison, Crow et al. 1992). Complex B is then converted into the active spliceosomal complex C after a conformational rearrangement. Complex C consists of U2, U5 and U6, while U1 and U4 snRNPs are destabilized during the conformational rearrangement (Lamond, Konarska et al. 1988) (Lamond 1995). At the same time, the first step of splicing occurs. The phosphodiester bond is broken at the junction of the exon and 5' splice site and lariat is formed (Teigelkamp, McGarvey et al. 1994; Roy, Kim et al. 1995). This step is initiated by U6 snRNP interaction with the 5' splice site and U2 interaction with the branch site positions the spliceosome (Sun and Manley 1995). After the first step of splicing, a second conformational change occurs, resulting in the release of the lariat intron and ligated exon products.

1.2 ALTERNATIVE SPLICING

How can the human genome, with only the approximately 25,000 protein-coding genes, encode many more proteins (Xie 2008) (I.H.G.S 2004)? The answer is alternative splicing. The pre-mRNA of most eukaryotic genes contains both exons and introns. The joining of the exons in pre-mRNA can occur in multiple ways during RNA splicing, termed alternative splicing. Typically, the splicing pattern of a multi-exon mRNA can be altered in many ways to generate multiple mRNAs, encoding distinct protein isoforms (Figure 2). The alternative splicing was first observed in adenoviruses, which produce two different coding mRNA from the same pre-mRNA due to different splicing pattern (Berget, Moore et al. 1977; Chow, Gelinas et al. 1977). Later on, alternative splicing was also found in cellular genes (Early, Rogers et al. 1980). Since then, more and more genes have been found to be alternatively spliced. One extreme example of alternative splicing was the Dscam gene from *Drosophila*, which in theory can produce a staggering 38,016 different mRNAs (Schmucker, Clemens et al. 2000). In fact, it has been found that alternative splicing is ubiquitous in eukaryotes (Black 2003). In human, it is now estimated that about 95% of multi-exon genes undergo alternative splicing, much more than previously estimated (Pan, Shai et al. 2008) (Johnson, Castle et al. 2003). Therefore, alternative splicing is a major contributor to protein diversity in metazoan organisms.

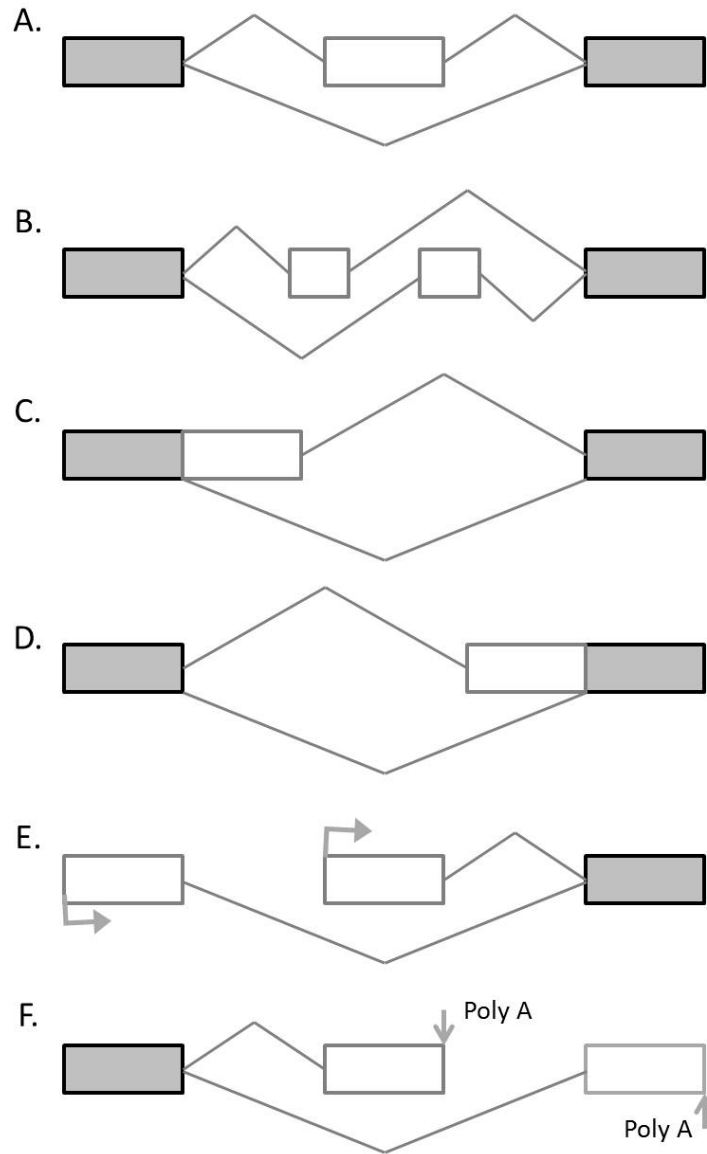


Figure 2. Patterns of alternative splicing.

Constitutive sequences are in gray boxes. Alternative segments are in white boxes. (A) Alternative spliced exon can be either included or excluded in the mRNA. (B) Mutually exclusive exons. (C, D) Alternative 5' or 3' splice sites. (E, F) Alternative promoters and alternative poly(A) sites.

Although the consensus sequence for splicing is known, the information from these consensus splice sites is not sufficient for accurate splicing (Lim and Burge 2001). Therefore, accessory factors are required to define the proper splicing sites. Generally, there are two groups of accessory factors, enhancers and silencers. Enhancers usually function to aid the assembly of spliceosome; while silencers function to block the spliceosome from accessing to the 5' and 3' splice sites. Proper alternative splicing is a balancing result from of these enhancers and silencers. In metazoans, spliceosome assembly is regulated by multiple pathways, leading to an alternatively spliced exon inclusion or skipping. Therefore, the tissue and developmental specific expression of splicing enhancers and silencers tunes splicing patterns in a spatial and temporal manner.

Alternative splicing is highly regulated. A single nucleotide change in splice sites or cis-acting splicing regulatory sites may lead to differences in the splicing pattern of a single gene. These splicing pattern changes in humans could cause diseases. In cancer cells, it has been shown there is a high proportion of abnormally spliced mRNA. Although it is not clear whether the abnormal splicing is a result of cancer or contributes to cancer, it has been shown that there is a reduction of alternative splicing in cancerous cells compared to normal cells (Skotheim and Nees 2007). In some cases, abnormal splicing may cause or contribute to human disease directly. It has been reported that autosomal dominant forms of retinitis pigmentosa is caused by mutation in the splicing factors PRPF31/U4-61k and PRP8 (Vithana, Abu-Safieh et al. 2001; Boon, Grainger et al. 2007; Wilkie, Vaclavik et al. 2008). Moreover, splicing errors induced by genetic mutations are responsible for protein defects and are found in human diseases. A probabilistic analysis indicates that over 60% of human disease-causing mutations affect splicing rather than

directly affecting coding sequences (Lopez-Bigas, Audit et al. 2005). Therefore, understanding regulation of alternative splicing will help to develop gene therapy to treat human diseases.

1.2.1 Cis-regulatory RNA Elements

Most weakly spliced exons are regulated by additional regulatory cis-acting elements and trans-acting factors. The cis-acting elements are generally divided into enhancers which include exonic splicing enhancers (ESE) and intronic splicing enhancers (ISE), and silencers which include exonic splicing silencers (ESS) and intronic splicing silencers (ISS). ESE and ESS are regulatory elements located within the exons that they regulate; while ISE and ISS are regulatory elements located in the flanking intron sequences in close proximity to the exons that they regulate (Black 2003). These RNA elements are typically short and present in multiple copies. These short regulatory RNA elements provide additional information for the splicing machinery to define the splice sites and are necessary to specify alternative splicing patterns.

Most ESEs contain purine-rich sequences, such as (RAR)_n, and play positive roles in both constitutive and alternative splicing. These RNA elements are bound by SR-family splicing factor, promoting the utilization of adjacent splice site. Many ESEs have been identified using SELEX (Liu, Zhang et al. 1998; Liu, Chew et al. 2000). The ESEs for ASF/SF2, SC35, SRp40 and SRp55 have been obtained and used to generate ESEFinder (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese finder.cgi>) (Fairbrother, Yeh et al. 2002; Cartegni, Wang et al. 2003). Separately, Fairbrother *et al.* has developed a program to predict the ESE using computational method (<http://genes.mit.edu/fas-ess/>) (Fairbrother, Yeh et al. 2002). Contrary to ESEs, ESSs

usually repress exon splicing by inhibiting the splicing machinery assembly via recruiting heterogeneous nuclear ribonucleoproteins (hnRNP) proteins (Amendt, Si et al. 1995; Zhu, Mayeda et al. 2001; Domsic, Wang et al. 2003). For example, hnRNP I/PTB bind to many exonic splicing silencers, blocking access to the splicing machinery through protein multimerization (reviewed by (Wagner and Garcia-Blanco 2001)). Using a cell-based splicing reporter system, Wang *et al.* has systematically screened the ESSs in human cells, which are available online (<http://genes.mit.edu/fas-ess/>) (Wang, Rolish et al. 2004). Intronic RNA regulatory elements, intronic splicing enhancers (ISE) and intronic splicing silencers (ISS), are also involved in the control of alternative splicing. One example of ISS is a polypyrimidine tract at the 3' splice site. The consensus sequences are UCUU or CUCU repeats in either the upstream or downstream intron. The polypyrimidine tract represses splicing by recruiting polypyrimidine tract binding protein (PTB) (Mullen, Smith et al. 1991; Ashiya and Grabowski 1997). ISEs in the intron usually promote the adjacent exon splicing. One example of ISE is the CUG repeats from intronic muscle-specific splicing enhancers (MSEs) of cardiac troponin T (cTNT) exon 5. Enhancement of proximal upstream exon splicing by this ISE is mediated by CUG binding protein (CUGBP) (Ryan and Cooper 1996; Philips, Timchenko et al. 1998). However, when CUGBP2 motifs are found in the upstream intron surrounding the branch site, binding of CUGBP2 represses the downstream exon splicing (Dembowski and Grabowski 2009).

The regulatory elements are useful in predicting splicing pattern. One of the important long-term goals in the splicing field is to determine the code for splicing that will be able to predict the splicing pattern by sequence inspection alone. However, the roles of these regulatory elements are influenced by the expression levels and activation status of splicing factors, which may vary in different cells or during different development stages. Another issue is the copy

number of the regulatory motifs. A single motif that has a weak effect on splicing can have a strong effect in the presence of additional copies of the same or functionally similar motifs (Black 2003). In addition, combinations of ESE/S and ISE/S motifs are often be used to fine tune the splicing, which may make the prediction even harder.

1.2.2 Trans-acting Proteins

The cis-acting splicing enhancers and silencers in pre-mRNAs interact with trans-acting factors controlling alternative splicing. These trans-acting factors, including serine/arginine-rich (SR), heterogeneous nuclear ribonucleoproteins (hnRNP), and hnRNP K-homology (KH) families, regulate splicing at the level of spliceosome function. These splicing factors are recruited by their RNA binding motif (ESE/S or ISE/S elements) in the pre-mRNA. Observed splicing patterns are often a result of the dynamic balance of enhancing and silencing functions.

SR proteins generally promote exon splicing. These factors are not only involved in alternative splicing but also involved in constitutive exon splicing. Upon binding to ESE motifs, SR proteins recruit factors such as U1 snRNP or U2AF and promote spliceosome assembly, or antagonize the effects of splicing silencers to facilitate exon definition. SR family proteins contain members of SRp20, SRp35, SF2/ASF, SC35, SRp30c, SRp40, SRp46, SRp54, SRp55, SRp75 and 9G8 (Graveley 2000). All SR proteins contain an N-terminal RNA recognition motif (RRM) and a C-terminal RS domain (Figure 3A). The RRM domain is mainly responsible for RNA binding. By targeting to particular exonic elements, the RRM domain determines the specificity of SR proteins. The RS domains mainly function as a protein interaction domain and

they are largely interchangeable (Chandler, Mayeda et al. 1997; Wang, Xiao et al. 1998). Switching the RS domains of different SR proteins maintain their activity both in vitro and in vivo. In addition, tethering the RS domain to exon is sufficient to activate exon splicing in vitro (Graveley and Maniatis 1998). The un-phosphorylated RS domain is highly positively charged and may be involved in protein RNA interaction. However, phosphorylation is required for the RS domain mediated protein/protein interactions (Mermoud, Cohen et al. 1994; Prasad, Colwill et al. 1999; Sanford and Bruzik 1999). It has been reported that SR protein kinase (SRPK1, SRPK2) activities are required for RS domain to mediate the RS proteins interaction (Wu and Maniatis 1993).

Generally, the hnRNP family proteins are characterized by their association with unspliced mRNA precursors (hnRNA). hnRNPs comprise a large family of proteins. There are more than 20 major proteins were resolved by two-D gel in human (Dreyfuss, Matunis et al. 1993). Proteins in the hnRNP family contain multiple RNA recognition motifs (RRMs) and often promote exon skipping by antagonizing the roles of SR proteins or snRNPs. One well-studied example is the polypyrimidine tract binding protein (PTB, or hnRNP I), which binds to pyrimidine-rich elements in the RNA and thereby mediates splicing repression in a long list of alternatively spliced pre-mRNAs (reviewed by (Sawicka, Bushell et al. 2008)). However, in some cases, some hnRNP family proteins may promote splicing, such as Fox1 and Fox2 (Underwood, Boutz et al. 2005). Others hnRNP family proteins may have dual roles capable of both enhancement and silencing, such as hnRNP H (Han, Yeo et al. 2005). In the KH domain family, the Nova1 and Nova2 splicing factors have been characterized as dual-functional regulators of alternative splicing events for transcripts encoding synaptic functions (Ule, Jensen et al. 2003; Ule, Ule et al. 2005).

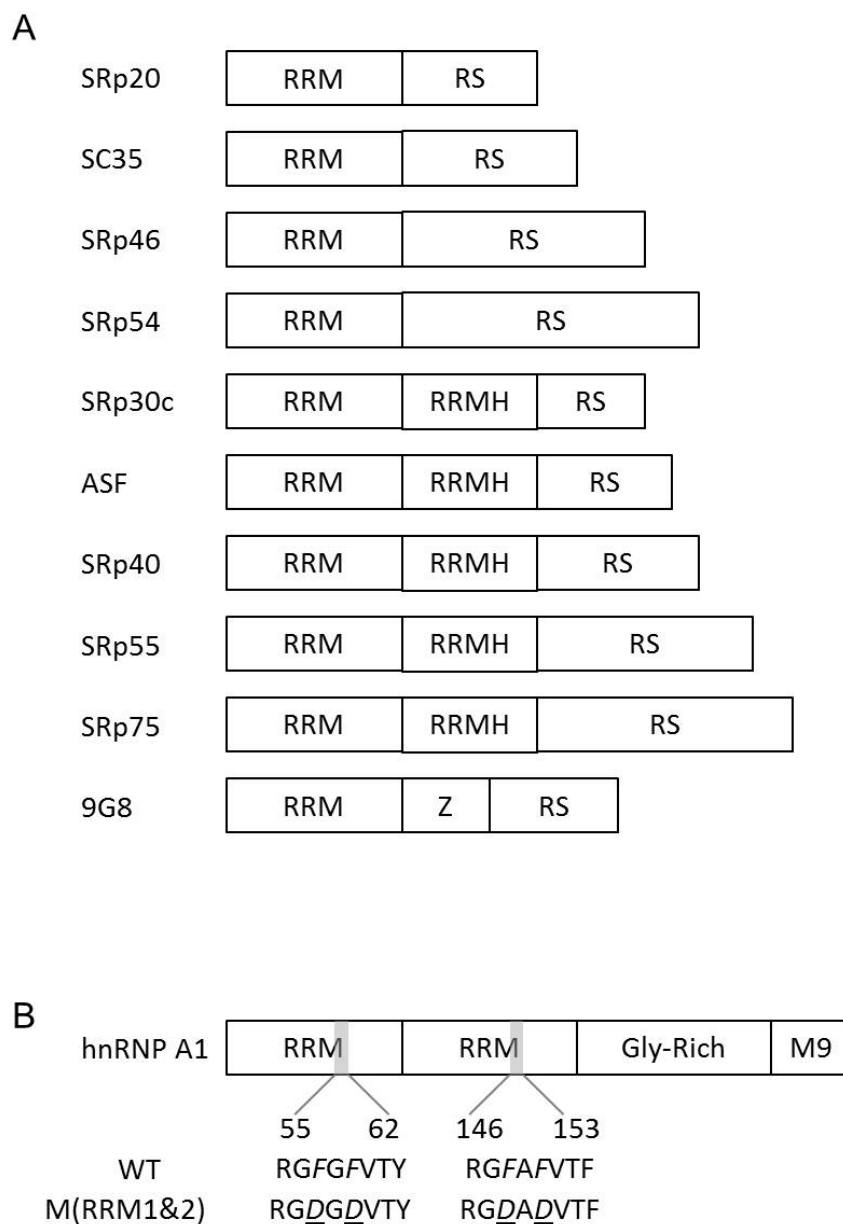


Figure 3. Schematic structure of human SR proteins and hnRNP A1

(A) The domains structures of the known members of the human SR protein family. RRM: RNA recognition motif; RRMH: RRM homology; Z: zinc knuckle, RS: arginine/serine-rich domain (Graveley 2000). (B) hnRNP A1 contains two RRM domains at the N-terminal, followed by a Gly-rich domain. M9 is at the C-terminus. Critical sequences for RNA binding are shown (WT). Sequence of mutant M(RRM1&2) with F57D and F59D in RRM1, and F148D and F150D mutations is also indicated.

1.2.3 hnRNP A1

hnRNP A1 is the most widely studied hnRNP protein. It was firstly identified as a splicing factor that counteracted SR proteins in an in vitro assay of splice site shifting. In the assay, 5' splice site selection was determined by the relative concentration of hnRNP A1 and SF2. More hnRNP A1 favors the distal 5' splice site, whereas more SF2 favors proximal 5' splice sites (Mayeda and Krainer 1992; Mayeda, Helfman et al. 1993). Since then, hnRNP A1 has been shown to bind to ESSs in the HIV, FGFR2, NMDAR and other transcripts (Caputi, Mayeda et al. 1999; Del Gatto-Konczak, Olive et al. 1999; An and Grabowski 2007) (Han, Yeo et al. 2005).

hnRNP A1 contains two RRM domains and a glycine-rich domain (Figure 3B). A crystal structure of the two RRM domains or UP1 of hnRNP A1 bound to the telomere repeat DNA d(TTAGGG)₂ shows that UP1 dimer binds to two strands of DNA and each strand contacts RRM1 of one monomer and RRM2 of the other (Ding, Hayashi et al. 1999). This result indicates that hnRNP A1 might bind to the RNA elements in the pre-mRNA in a similar way, which would allow cooperative binding to multiple splicing silencer elements and looping of the RNA between binding elements (Black 2003). Similar to the SR proteins, the RRM domain and glycine-rich domain of hnRNP A1 are modular in function. The RRM domains are mainly responsible for RNA binding, while the glycine-rich domain is involved in protein/protein interactions (Dreyfuss, Matunis et al. 1993). Tethering just the glycine-rich domain is sufficient to repress K-SAM exon splicing (Del Gatto-Konczak, Olive et al. 1999).

Several mechanisms have been proposed for A1-mediated splicing repression, and its mechanism may differ between different transcripts. One of the proposed models is that hnRNP A1 could interfere directly with the assembly of spliceosomal components by creating a

silencing zone on the pre-RNA. This model is well demonstrated by the splicing of Tat exon 3. Using an in vitro splicing system, the Krainer lab found that addition of A1 specifically inhibited SC35- but not SF2/ASF-activated splicing (Zhu, Mayeda et al. 2001). It was found that in addition to binding the ESS, hnRNP A1 crosslinked to exonic RNA distal to the ESS in the region of the SR protein binding sites. This crosslinking occurred only if the ESS was present. This result shows that hnRNP A1 binding at the ESS nucleates the cooperative non-specific binding of A1 upstream, thus creating a silencing zone on the pre-mRNA and blocking the assembly of spliceosomal components. In addition, as indicated the crystal structure with the telomere DNA, hnRNP A1 could form dimer or multimer and repress exon splicing by looping up the exon. This model is demonstrated by the splicing of exon 7B of hnRNP A1 itself (Blanchette and Chabot 1999; Nasim, Hutchison et al. 2002).

hnRNP A1 is one of the most abundant nuclear proteins in mammalian cells. It is not only involved in a variety of RNA-related processes including alternative RNA splicing and mRNA transport (Izaurralde, Jarmolowski et al. 1997), but is also involved in telomere and microRNA precursor biogenesis (LaBranche, Dupuis et al. 1998; Guil and Caceres 2007; Michlewski, Guil et al. 2008). Telomeric DNA of mammalian chromosomes consists of long tandemly repeated sequences, and maintaining the integrity of these repeats is essential for cell survival. hnRNP A1, which is involved in promoting telomere access by interacting with telomeres, may modulate telomere length (reviewed by (Ford, Wright et al. 2002)). It has been shown that hnRNP A1 directly bind to the single-stranded TAGGGT repeats, which is similar to its consensus RNA binding motif, UAGGGA/U. Moreover, deficiency in hnRNP A1 expression in a mouse erythroleukemic cell line is associated with short telomeres. Importantly by restoring hnRNP A1 expression in cells, the length of telomeres was increased (LaBranche, Dupuis et al.

1998; Fiset and Chabot 2001). In addition to its roles in maintaining telomere length, hnRNP A1 has been shown to act as an auxiliary factor for the Drosha-mediated processing of a microRNA precursor, pri-miR-18a. hnRNP A1 binds to the pri-miR-18a terminal loop and to a region within the stem and rearranges the RNA secondary structure, allowing efficient processing by the Drosha/DGCR8 complex (Guil and Caceres 2007; Michlewski, Guil et al. 2008).

1.2.4 Regulation of Splicing Factors Activity

Alternative splicing is highly regulated by splicing factors. Therefore activities of splicing factors also need to be regulated in a tissue- or developmental dependent manner. In the mouse brain, neural PTB (nPTB) is exclusively expressed in neurons, while PTB expression is only expressed in precursor cells (NPC), glia, and other nonneuronal cells (Boutz, Stoilov et al. 2007). The reason for this mutually exclusive pattern of expression is that PTB causes skipping of an exon in the nPTB transcript, resulting in nonsense-mediated mRNA decay (NMD) of the transcript. Recently, it has been shown that the activity of splicing factors is also regulated in response to external stimuli, such as growth factors, cytokines, hormones and depolarization. One example is the regulation of protein kinase C (PKC) β II alternative splicing in response to insulin. Application of insulin to myotubes results in the incorporation of the PKC β II exon within 30 minutes of stimulation (Chalfant, Watson et al. 1998). Binding of insulin activates PI3-K via binding to the insulin receptor substrate (IRS). PI3-K forms phosphatidylinositol 3,4,5-trisphosphate, which ultimately phosphorylates SRp40. Activation of SRp40 results in exon β II inclusion (Patel, Chalfant et al. 2001). In addition, it has also been reported that stress stimuli such as osmotic shock or UVC irradiation causes some SR proteins and hnRNP A1 to leave the

nucleus and accumulate in the cytosol. As a result, the splicing patterns of reporter genes are changed by the stress stimuli (van der Houven van Oordt, Diaz-Meco et al. 2000).

hnRNP A1 is involved in a variety of RNA-related processes including alternative RNA splicing and mRNA transport. Recent studies have implicated hnRNP A1 as a splicing factor that mediates induced changes in alternative splicing in response to external stimuli. Osmotic stress treatment of cells results in serine-specific phosphorylation within a C-terminal peptide, leading to the increase of cytoplasmic hnRNP A1 (van der Houven van Oordt, Diaz-Meco et al. 2000; Allemand, Guil et al. 2005). More recently, An and Grabowski have shown increased binding of hnRNP A1 to C1 exon UAGG motif in the nuclear extract from depolarized neurons suggesting that KCl induced depolarization might change hnRNP A1 RNA binding affinity (An and Grabowski 2007). However, it is not clear how the activity of hnRNPA1 is modified in response to cell excitation. Phosphorylation is one of major mechanisms that regulate alternative splicing in response to a stimulus (Stamm 2002). hnRNP A1 has been shown to function as a substrate for protein kinase C (PKC), protein kinase A (PKA), and casein kinase (Idriss, Kumar et al. 1994). The PKA phosphorylation site has been mapped to Ser199 in vitro. Mutation of Ser199 to Pro abolishes PKA phosphorylation of hnRNP A1 (Cobianchi, Calvio et al. 1993; Jo, Martin et al. 2008). It has been shown that hnRNP A1 can also be phosphorylated by AKT at S199. Phosphorylation of hnRNP A1 by AKT has been shown to inhibit internal ribosome entry site (IRES)-mediated translation initiation by blocking its strand annealing (Martin, Masri et al.; Jo, Martin et al. 2008). In addition, the silencing effect of hnRNP A1 on the splicing of CD44 v5 exon was attenuated by overexpression of a constitutively active form of the mitogen-activated protein/ERK kinase kinase 1, and by the small GTP-binding protein Cdc42, indicating that hnRNP A1 is regulated by these kinases (Matter, Marx et al. 2000). In addition to

phosphorylation, hnRNP A1 may also undergo methylation, sumoylation and ubiquitination (Liu and Dreyfuss 1995; Iervolino, Santilli et al. 2002; Zhang and Cheng 2003; Li, Evdokimov et al. 2004). Taken together, these results suggest that the activity of hnRNP A1 could be regulated in response to various biological stimuli.

1.3 SIGNAL TRANSDUCTION AND ALTERNATIVE SPLICING

Alternative splicing is now recognized as a ubiquitous mechanism to control gene expression in a tissue- and developmental-specific manner. Growing evidence from the past few years also highlights the existence of networks of signal-responsive alternative splicing in a variety of cell types. While the mechanisms by which signal transduction pathways affect the splicing factors are relatively poorly understood. A few studies have shown how extracellular stimuli can be communicated to splicing factors that control splice site selection by the spliceosome.

1.3.1 Ca^{2+} /calmodulin-dependent Protein Kinase IV

Calcium is a universal second messenger in all eukaryotic cells. It regulates many activities by forming a complex with calmodulin (CaM), which is a 17-kDa acidic protein with four high-affinity Ca^{2+} binding motifs. Binding of Ca^{2+} increases CaM binding affinity for its targets, the Ca^{2+} /CaM-dependent kinase (CaMK) family (Soderling 1999). CaMK IV is one the member of CaMK family. It contains an N-terminal kinase domain, an auto-regulatory domain and an overlapping CaM-binding domain. Bound Ca^{2+} /CaM activates CaMK IV by removing the autoregulatory domain from the catalytic pocket to enable substrate access. For full activation, CaMK IV requires phosphorylation by CaMKK (Racioppi and Means 2008). CaMK IV plays important roles in the expression of genes required for cellular processes such as long-term

potentiation and T-cell activation by phosphorylating and activating transcription factors such as CREB (Anderson and Kane 1998). Thus, CaMK IV serves as a critical mediator of Ca^{2+} induced gene transcription in both neurons and T-cells.

A few recent studies have shown CaMK IV is involved in mediating alternative splicing in response to external stimuli. The role of CaMK IV in regulating alternative splicing was first reported by Xie and Black. They reported that alternative splicing of Slo (BK potassium channel) transcripts was regulated by depolarization of the excitable pituitary cell line GH3 in a CaMK IV-dependent pathway. Further investigation identified a 3' splicing site intronic CaMK IV-responsive RNA element (CaRRE) motif, which was required and sufficient to mediate the CaMK IV induced splicing silencing (Xie and Black 2001). Later on, the CaRRE had been defined more precisely to a 11-nucleotide motif located between the polypyrimidine tract and the 3' AG within the STREX 3' splicing site (Xie, Jan et al. 2005). Several other exons that carry this element are also regulated by CaMK IV, suggesting that this might be a general mechanism. More recently, both An and Lee showed that depolarization induced C1 cassette splicing silencing of NMDA receptor is also mediated by CaMK IV (An and Grabowski 2007; Lee, Xing et al. 2007). Treatment of cells with CaMK IV inhibitor KN93 attenuated depolarization induced C1 splicing silencing. Over-expression of constitutively active CaMK IV led to repression of C1 splicing. Moreover, two CaRRE motifs were identified in the C1 exon (Lee, Xing et al. 2007). Together, these results suggest that depolarization induced splicing silencing is mediated by CaMK IV and CaRRE cis-elements in the RNA. However, the trans-factor that mediates CaMK IV and the CaRRE cis-elements still remain elusive (review in (Blaustein, Pelisch et al. 2007)).

1.3.2 Protein Kinase A

The Protein Kinase A or cAMP-dependent protein kinase (PKA) pathway ubiquitously regulates many cellular processes such as metabolism, cell growth and differentiation and synaptic neurotransmitter release. In the absence of cAMP, PKA is a holoenzyme composed of two catalytic (C) subunits bound by a regulatory (R) subunit dimer. cAMP binding to the R subunits dissociates the holoenzyme and the released C subunits phosphorylate a number of cytoplasmic substrates. A portion of the C subunits also translocate into the nucleus, where the major known targets of nuclear C subunits are a group of cAMP-responsive nuclear factors called CREBs (cAMP-response element binding proteins), which bind and regulate the transcription of genes containing cAMP-response elements (CREs).

Recently, a few studies have implicated the roles of PKA in regulating alternative splicing. Kvissel and colleagues have shown that after activation, the C subunit of PKA translocates to the nucleus and co-localizes with splicing factor compartments (SFCs) (Kvissel, Orstavik et al. 2007). Further investigation showed that activation of PKA can phosphorylate a variety of splicing factors, including several members of the SR-protein family in vitro. In vivo splicing assay also showed that PKA can change the splicing pattern of E1A splicing reporter (Kvissel, Orstavik et al. 2007). In addition, Xie and Black have reported that PKA regulates the nucleo-cytoplasmic transport of PTB/hnRNP I, a splicing factor with important functions in alternative pre-mRNA splicing, viral RNA translation, and mRNA localization (Xie, Lee et al. 2003). PKA directly phosphorylates PTB on a conserved Ser16, resulting in the accumulation of PTB in the cytoplasm and implicating PKA in regulating the activity of PTB (Xie, Lee et al.

2003). More recently, Li and Xie have identified a PKA response elements CAAAAAA motif using SELEX method (Li, Liu et al. 2009). The identified CAAAAAA motif is sufficient for the PKA induced splicing silencing of exon 16 of the CaMK kinase β 1. Together, these results suggest that PKA might play important roles in regulating alternative splicing in response to external stimuli. However, the mechanisms by which the activity of splicing factors is affected remain unclear.

1.4 NMDA RECEPTOR

Alternative splicing in the nervous system greatly expands protein diversity, which is necessary for neural development and shaping the synapse for processes such as learning and memory. N-methyl-D-aspartate receptors (NMDARs) are excitatory neurotransmitter receptors, belonging to the subclass of ionotropic glutamate receptors. They are present at many excitatory synapses and are widely distributed in the mammalian brain (Laurie, Putzke et al. 1995; Cull-Candy, Brickley et al. 2001). These receptors allow calcium influx that regulates signaling pathways involved in synaptic plasticity in response to the binding of glutamate and glycine under depolarizing conditions. Their surface expression plays a crucial role in neuronal development of the brain, synaptogenesis, synaptic plasticity, and excitotoxicity (Zukin and Bennett 1995). The NMDA receptor forms a heterotetramer between two NR1 and two NR2 subunits. Alternative splicing of the NR1 subunit of NMDAR has been shown to modulate receptor function in a brain region-specific manner and in response to cell excitation.

1.4.1 NMDA Receptor NR1 subunit Alternative Splicing

Alternative splicing of three exons (N1, C1 and C2) of the NR1 subunit of the NMDA receptor pre-mRNA (GRIN1) generates eight receptor isoforms (Zukin and Bennett 1995; Cull-Candy, Brickley et al. 2001). The N1 cassette exon (Ensembl transcript GRIN1-002, exon 3b), which encodes a portion of the extracellular domain at the N-terminal, modulates receptor sensitivity to zinc ions, protons, and polyamines. Mutually exclusive splicing of C2 and C2' exons adjusts receptor trafficking to the synapse (Mu, Otsuka et al. 2003). In addition, The C1 cassette exon (GRIN1-002, exon 19), which encodes an intracellular region at the C-terminal, regulates a variety of activities of the NMDA receptor, including trafficking from the ER to plasma membrane, phosphorylation by protein kinase C (PKC) and protein kinase A (PKA), interactions with yotiao, neurofilament-L, and calmodulin, and activation of NMDAR-induced gene expression (Ehlers, Zhang et al. 1996; Tingley, Ehlers et al. 1997; Bradley, Carter et al. 2006) (Ehlers, Fung et al. 1998; Lin, Wyszynski et al. 1998). Thus, NMDA receptor structure and function are fine-tuned by alternative splicing.

Given its importance to neuronal functions, the C1 cassette in the NR1 subunit is a valuable model to study the splicing regulation. The C1 exon is predominantly included in the forebrain and predominantly skipped in the hindbrain (Zhang, Liu et al. 2002). Several SR proteins as well as RNA binding proteins Napor/CUPBP2, hnRNP H, and NOVA2 are thought to positively regulate this exon (Zhang, Liu et al. 2002; Han, Yeo et al. 2005; Ule, Ule et al. 2005). Identified splicing enhancers for C1 exon include binding sites of SR protein ASF/SF2, SC35, and SRp40 within the exon, and binding sites for hnRNP H and Napor/CUGBP2 in the downstream intron (Figure 4A). In contrast, hnRNP A1 can repress the splicing of this exon via

its interactions with two exonic UAGG motifs and one intronic G tract (Han, Yeo et al. 2005). The interactions between these many different proteins and RNA motifs are thought to contribute to the complex pattern of splicing seen for C1 within the brain.

Recent studies have shown that the C1 cassette is an inducible exon in neuronal cells. Treatment of cells with KCl to induce cell depolarization will change C1 splicing pattern to predominantly exon skipping. However, the molecular links that mediate the signal transduction from depolarization on the membrane to the splicing events in the nucleus are largely unclear. Previous studies from our lab have shown that treatment of cells with CaMK IV and PKA inhibitor KN93 and H89 will attenuate KCl induced splicing skipping, suggesting that CaMK IV and PKA pathway might be involved in the regulation of KCl induced C1 splicing skipping (An and Grabowski 2007). Furthermore, two types of CaMK IV-responsive RNA elements (CaRREs) have been found within the C1 exon (Lee, Xing et al. 2007). One element is similar to an element defined previously in the 3' splice site region of the responsive STREX exon of the BK channel (Xie and Black 2001). Over-expression of constitutively active CaMK IV with C1 splicing reporter results in the repression of C1 splicing. More importantly, the CaRREs motifs are transferable to other exons that are not regulated by CaMK IV (Lee, Xing et al. 2007). These findings suggest that one or more signaling pathways from NMDA receptors located at the membrane to factors in the nucleus are responsible for inducing changes at the level of splicing of the C1 exon. However, the splicing factors that mediate the signal pathways and alternative splicing remain elusive.

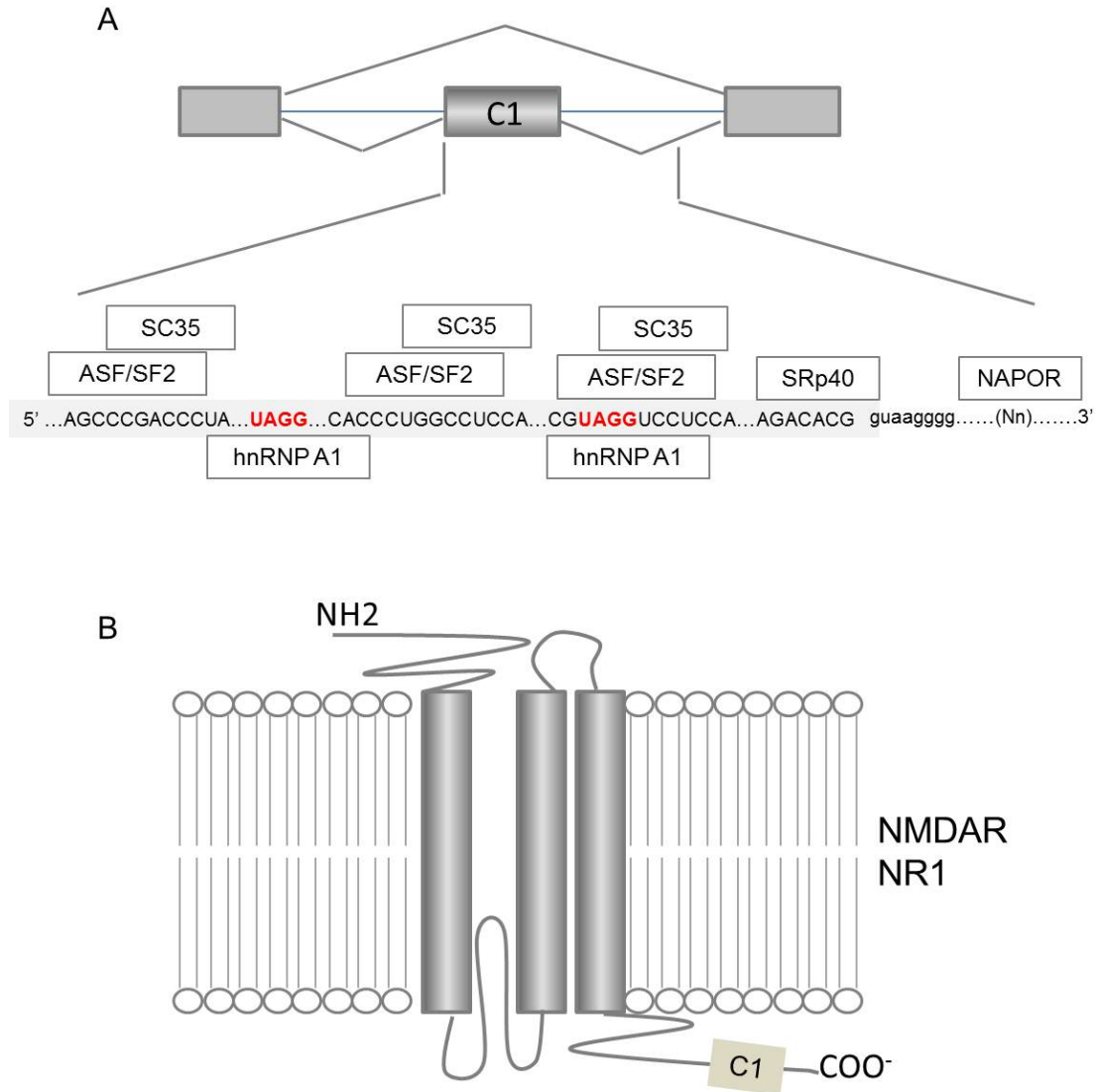


Figure 4. Schematic structure of C1 exon and NMDA receptor NR1 subunit

(A) C1 exon is alternative spliced. Below is a summary of splicing regulatory motifs. The regulatory splicing factors are also indicated. (B) Schematic structure of NMDA receptor NR1 subunit. The relative position of C1 cassette is shown.

hnRNPA1 has been implicated as the depolarization-responsive splicing factor involved in this signaling event. Previously, our lab has identified 2 UAGG motifs in C1 exon, which are repressed by splicing factor hnRNP A1 (Han, Yeo et al. 2005). Mutation of these motifs result in significant loss of hnRNP A1 induced C1 splicing silencing. Moreover, An and Grabowski have shown increased binding of hnRNP A1 to C1 exon UAGG motif in the nuclear extract from depolarized neurons suggesting that KCl induced depolarization might change hnRNP A1 RNA binding affinity (An and Grabowski 2007). However, it is not clear how the activity of hnRNPA1 is modified in response to cell excitation.

1.4.2 NMDA Receptor and PKA Pathway

The cAMP-PKA and Ca^{2+} signaling are crucial for synaptic plasticity in neurons. Ca^{2+} entry through NMDA receptor has been show to activate PKA pathway via cAMP (Grewal, Horgan et al. 2000). Meanwhile, the Ca^{2+} entry through the NMDA receptor is also under the control of cAMP-PKA signaling cascade (Skeberdis, Chevaleyre et al. 2006). It has been shown that PKA and protein phosphatase-1 (PP1) are associated with NMDA receptor NR1 subunit through a scaffold protein yotiao, which interacts with NR1 in a C1 exon-dependent manner (Lin, Wyszynski et al. 1998; Westphal, Tavalin et al. 1999). It has been proposed that PKA regulates NMDA receptors activity by phosphorylating the NMDA receptor or a receptor-associated protein, thereby inducing a conformational change of the NMDA channel pore and resulting in augmented Ca^{2+} influx; when PKA is inactive, the NMDA receptor or associated proteins are rapidly dephosphorylated by the PP1 (Skeberdis, Chevaleyre et al. 2006). Thus, the coupling of

PKA and PP1 to synaptic NMDA receptor by yotiao enables the bidirectional regulation of NMDAR channel activity by PKA.

1.4.3 NMDA Receptor and Disease

The NMDA receptor is a non-specific cation channel permeable to Ca^{2+} , Na^{+} , and K^{+} . It plays critical roles in excitatory synaptic transmission, plasticity and excitotoxicity in the neuronal cells. NMDA receptors are modulated by a number of endogenous and exogenous compounds and play a key role in a wide range of physiological (e.g. memory) and pathological processes (e.g. excitotoxicity) (Li and Tsien 2009). Dysregulation of NMDR receptor could result in disease in human beings.

Huntington's disease (HD) is a progressive neurological disorder, characterized by the symptoms of abnormal dance-like movements, cognitive disturbances, and disorders of mood (Levine, Cepeda et al.). Neuropathologically, HD is characterized primarily by neuronal loss in striatum and cortex, and specifically targets the medium-sized spiny striatal neurons (MSNs) (Cowan 2006). Early model of HD was established by the treatment of NMDA receptor agonist quinolinic acid, which produced degeneration of MSNs that replicated the human neuropathology (Coyle 1979). This suggests that dysregulation of NMDR receptor may contribute to the HD.

Recently, a dual role for NMDA receptors has been uncovered depending upon the location of the receptor to synaptic or extrasynaptic sites (Fan and Raymond 2007). Synaptic NMDA receptors activate cellular survival pathways while extrasynaptic receptors activate

pathways that lead to cell death. Milnerwood et al. showed that the balance between synaptic and extrasynaptic NMDA receptors is disrupted in MSNs in a mouse model of HD (Milnerwood, Gladding et al. 2010). Treatment with memantine, an NMDA antagonist which antagonizes extrasynaptic but not synaptic NMDA receptors at low dosages, reverses the decrease (Milnerwood, Gladding et al. 2010). Thus, the balance of activity or the numbers of each of the types of NMDA receptors at each location can sway the outcome between cell survival and cell death.

In addition, inappropriate activation of NMDARs has been implicated in the aetiology of several disease states. In particular, excessive calcium influx through NMDARs can cause excitotoxic neuronal death, and thus blockade of NMDARs is neuroprotective in animal models of both stroke and seizure (Hardingham and Bading 2003).

1.5 THESIS GOALS AND OBJECTIVES

Above, I have discussed the current status of research in the field of alternative splicing in details. One of the main questions that remain unanswered is how splicing machinery is regulated by signaling pathways. Specifically, what are the molecular links mediating the external stimuli and the splicing events in the nucleus? How are splicing factors regulated by the signal pathways? Here, the molecular links mediating KCl induced C1 splicing silencing are studied extensively to provide insight into some of these questions.

The C1 exon of the glutamate NMDA receptor NR1 subunit is alternatively spliced in a tissue-specific manner. It is predominantly included in the forebrain, and predominantly skipped in the hindbrain. The C1 exon encodes an intracellular region at the C-terminal, regulating a variety of activities of the NMDA receptor. Importantly, it has been reported C1 exon splicing is regulated by cell depolarization. Therefore, the C1 exon is a valuable system to study the signal transduction pathway of alternative splicing.

The main goal of this study is to identify the molecular links that mediate depolarization induced C1 splicing skipping. Specifically, the purpose of this study is (1) to identify the roles of Ca^{2+} in mediating depolarization induced C1 splicing silencing, (2) to identify the trans-acting factors that mediate CaMK IV signaling and C1 alternative splicing, (3) to identify the roles of PKA in mediating depolarization induced C1 splicing silencing, and (4) to determine whether hnRNP A1 is recruited to C1 splicing site in vivo during depolarization. To investigate the roles of Ca^{2+} in mediating depolarization induced C1 splicing silencing, Ca^{2+} imaging will be used monitor the Ca^{2+} concentration during depolarization. The roles of CaMK IV and PKA in regulating C1 splicing will be investigated by the splicing assay with constitutively active CaMK IV and PKA. Since the C1 splicing is regulated by hnRNP A1, the roles of hnRNP A1 in mediating CaMK IV and PKA C1 splicing will also be investigated. Finally, hnRNP A1 recruitment assay will be used to address if hnRNP A1 is recruited during depolarization. Results from these analyses will provide insight into the signal transduction of KCl induced C1 splicing silencing and can be used a platform for the future analysis of signal transduction between external stimuli and alternative splicing.

2.0 MATERIALS AND METHODS

2.1 PLASMID CONSTRUCTION

EYFP A1 or EYFP A1 M(RRM1&2) was constructed by inserting the open reading frame of hnRNP A1 or A1 M(RRM1&2) into a pEYFP N1 vector using restriction site BsrG I site. pET-A1 M(RRM1&2) was a gift from Dr. Akila Mayeda (Fujita Health University). Splicing reporters, Wild-type and UAGG mutant C1 cassette splicing reporters, Sirt1 and Ess19a were the same as described in (Han, Yeo et al. 2005; An and Grabowski 2007). Ess19a-AUGG was made by replacing the sequence AAGCTTGTTAGGTATAGGTACC in the exon of Ess19a with sequence AAGCTTGTATGGTAATGGTACC using restriction site Hind III and KpnI. Constitutively active PKA and dominant negative PKA were gifts from Dr. Jiuyong Xie (University of Manitoba). Constitutively active CaMK IV and dominant negative CaMK IV were gifts from Dr. Douglas L. Black (University of California, Los Angeles).

2.2 PRIMARY CORTICAL NEURONS CULTURE

Two dozen fresh E18 rat cerebral cortexes (hemispheres) were ordered from Zivic Miller. Tissue was first rinsed with 20 ml of 1x PBS for three times, and then digested with 20 ml of 1x trypsin in trypsin media (MEM (Gibco), 0.1% glucose (sterile filtered), 2 mM L-glutamine (sterile filtered, light sensitive), 20 mM HEPES (Sigma)) at 36°C for 20 minute. After digestion, trypsin was carefully removed and rinsed 3 times with 20 ml of trypsin media. Digested tissue was then re-suspended in 8 ml of trypsin media, transferred to 10 ml syringe, and passed through an 18 gauge needle 8-10 times to homogenize the tissue. Homogenized tissue was transferred to a 50 ml conical tube to let the connective tissue and debris settle down to the bottom. The supernatant was transferred to 15 ml falcon tubes, and the cells in the supernatant were spun down at 800-900 rpm for 3-4 minutes. Cell pellets were re-suspended in a total of 25 ml growth media (Neurobasal media (Gibco) supplemented with 0.5 mM L-glutamine (sterile filtered, light sensitive) and 2% B-27 supplement (Invitrogen)). Cells were counted and $6-10 \times 10^5$ cells were plated per well of 6-well plate. Dishes were pre-coated with poly-D-lysine (BD Biosciences). If coverslips were used, four poly-D-lysine-coated coverslips (BD Biosciences) were placed into each well of an uncoated 6-well plate, and $6-10 \times 10^5$ cells were plated per well. Half of the growth media was replaced after 4 days and primary cortical cultures were incubated in a 37°C tissue culture incubator with 6% CO₂. Primary cortical cultures contained mostly neurons and few supporting glial cells. To induce cell depolarization, 50 mM KCl was added to the growth medium.

2.3 CALCIUM IMAGING

To carry out calcium imaging experiments, neurons (DIV8) or N18TG2 cells on a coverslip were incubated with 2 μ M Fura 2AM in incubation buffer (140mM NaCl, 5mM KCl, 2mM MgCl₂, 2mM CaCl₂, 10 μ M HEPES pH7.4) for 30min followed by 10min wash in incubation buffer. Following the wash, coverlips were mounted in a chamber that allows rapid solution exchange. The sample was illuminated by 340 and 380 nm alternating light every 2 seconds and fluorescence was collected at 510 nm using a CCD cooled camera. The images were analyzed using Metafluor software and cytoplasm Ca²⁺ changes were expressed in arbitrary units reflecting fluorescence intensity induced by 340 nm divided by the intensity induced by 380 nm light as described before (Ca²⁺ imaging experiments were done under the direction of Dr. Kirill Kiselyov).

2.4 TRANSFECTION AND RNA ANALYSIS

N18TG2 cells were cultured in DMEM, 10% (v/v) fetal bovine serum (FBS). Twenty-four hours prior to transfection 1.5×10^5 N18TG2 cells were seeded on 6-well plates to achieve 60–80% confluency. Plasmid DNA (1.25 μ g) was transfected into N18TG2 cells using 2 μ l of Lipofectamine 2000 (Invitrogen) per well of a 6-well plate. Detailed transfection procedures were described in (An and Grabowski 2007). For splicing assay, 1 μ g splicing factors, CaMK IV or PKA and 0.25 μ g splicing reporter were used for transfection. Cells were harvested after 16-24

hours of transfection using Trizol (Invitrogen). For the transfection of the subunits of NMDA receptors for splicing assay, 0.3 µg NR1-4a, 0.6 µg NR2A and 0.25 µg C1 splicing reporter were used for transfection. For the transfection of the subunits of NMDA receptors for calcium imaging, 0.3 µg NR1-4a, 0.6 µg NR2A and 0.1 µg pEYFP-N1 were used for transfection (Qian, Buller et al. 2005). EYFP was used as a transfection reporter. About 50-70% transfection efficiency was achieved in N18TG2 cell. The primary reason for the N18TG2 was used for the most of the experiments is that there is no endogenous NMDA receptor.

For splicing pattern measurements, cells were harvested 24hours after transfection using TRIZOL (Invitrogen). Splicing patterns were measured by RT-PCR as describe in (An and Grabowski 2007). Reverse transcription (RT) reactions (20µl total volume) contained M-MLV reverse transcriptase (Invitrogen), 1µg RNA sample, and 0.5µg random primers (Promega). PCR reactions (10-µl volumes) contained 0.2 µM specific primers, 2 units of Taq DNA polymerase (Promega, Madison, Wisconsin, United States), 1/20th of the volume of the RT reaction, 0.2 mM dNTPs. Amplification was for 25 cycles. Primers were designed to amplify exon-included and skipped mRNAs in each sample. PCR products were resolved on 1.5% Agarose Gel containing 0.001% Sybr Green. Gel images were captured on Fuji LAS-3000 and quantitated using ImageGauge 4.0 software. Percentage of exon inclusion was calculated as Included/(included+skipped).

2.5 HNRNP A1/H KNOCKDOWN

To knockdown endogenous hnRNP A1/H in N18TG2 cells, N18TG2 cells were plated into 6-well plate at a concentration of 1×10^5 cells/well. After 24 hours plated, cells were transfected with 2 μ l A1 siRNA (40 μ M) using 4 μ l Lipofectamine 2000. 24 hours after siRNA transfection, cells were transfected with CaMK IV or PKA, together with C1 splicing reporter [8]. Cells were harvested 24 hours later for Western Blot or RT-PCR. The following sequence was used for hnRNP A1 knockdown: CAGCUGCGGAAGCUCUUCAUU. Antibody against A1 (4B10) used for Western blotting was a gift from G. Dreyfuss (University of Pennsylvania).

2.6 RNA FISH AND IMMUNOFLUORESCENCE

Cells on coverslip were washed with PBS twice, and then fixed with freshly made 3.7% paraformaldehyde in PBS (phosphate buffered saline) for 10 min at room temperature. After fixation, cells were washed with PBS and then permeablized with 0.2% Triton X-100 in PBS for 5min at room temperature, followed by washing with 50% ethanol. Cells were kept in 70% ethanol at 4°C.

Exon/Intron junction DNA/LNA (Locked nucleic acid) probe ctCttCccCttAccGtgTctTtgGa (LNA in capital letter) was synthesized by Sigma. DNA intron probe (300bp, downstream of C1 exon) was PCR amplified from rat genome DNA with primers GTAAGGGGAAGAGC

ACCCCAG and CTGACCAACATGACACAGCTTC. LNA (Locked nucleic acid) or DNA probes were labeled with Alexa Fluor 594 using ULYSIS nucleic acid labeling kit (Invitrogen). To prepare for the hybridization cocktail (modified from (Misteli, Caceres et al. 1998)), 1.5ul labeled probes were mixed with 0.5ul ssDNA (10mg/ml), 1ul *E.coli* tRNA (5mg/ml) and 2ul formamide, and then denatured at 80°C for 10min. After cooled down at room temperature, probes were mixed with 5ul 2× Hybridization Buffer (4x SSC (saline-sodium citrate), 4ug/ul BSA (Bovine serum albumin), 20% dextran sulfate (Sigma, MW 8000), 0.1U/ul RNasin, 20mM VRC (vanadyl-ribosyl complex, NEB)). To carry out RNA FISH incubation, cells on coverslips were rehydrated in 24-well plate with 500ul 50% ethanol, 1xPBS and 2xSSC for 2 min each before incubating with 10ul of hybridization cocktail overnight at 37°C. Inverted coverslips were washed with 30% formamide in 2× SSC at 37°C for 10 min twice, 2x SSC at RT for 5min twice, 1x SSC at RT 5min twice and 1x PBS at RT for 5min. Coverslips were mounted onto slide with 2ul of mounting solution (30% Glycerol, 12% Mowiol, 3% DABCC in 0.2M Tris-HCl pH 8.5) and stored in the dark at -20°C.

For detection of endogenous splicing factors, immunofluorescence was performed after RNA-FISH with additional fixation in 3.7% paraformaldehyde in PBS for 8 min at room temperature. Anti-A1 (9H10, Abcam) at 1:2,000, anti-ASF/SF2 (Abcam) at 1:500 and anti-SC35 (Sigma) at 1:500 in blocking buffer (1% Normal Goat Serum in PBST (0.1% Tween in PBS)) were used. Cells were incubated with primary antibodies at room temperature for 2 hours, followed by 3 washes with PBST, and then incubated with fluorescently labeled secondary antibodies (1:500) for 1.5 hours at room temperature, followed by 3 PBST washes. Coverslips were mounted and stored as the same as above.

2.7 FLUORESCENCE MICROSCOPY AND QUANTITATIVE RECRUITMENT ASSAY

Images were acquired on Zeiss LSM confocal laser scanning microscope using simultaneous scans to avoid shift between the two optical channels. The 488-nm laser line was used for detection of EYFP (for EYFP-A1) or Immunofluorescence (for endogenous splicing factors) signals and the 568-nm line for detection of Alexa 594 RNA-FISH signals. Images were analyzed using Image J software with colocalization and color-profiler plugins. Recruitment was detected as accumulation of EYFP/IF signal at the site of C1 transcription similar to the procedure that was previously described (Mabon and Misteli 2005). Positive recruitment was defined by two simultaneous criteria: a) the splicing factor signal was accumulated at the transcription site more than 1.5-fold above nucleoplasmic signal, and b) at least half the splicing factor intensity peak overlapped with at least half of the RNA-FISH intensity peak. All quantitations were performed on 8-bit grayscale images with no saturated pixels and no prior adjustment of the images. A total of 80 foci (~27 foci/experiment) from at least three experiments were quantitated for each splicing factor. Statistical analysis was performed using a standard Student t-test. For the splicing factors of ASF/SF2 and SC35, enrichment of the IF signal of >2 fold above its average nuclear intensity was defined as recruitment.

2.8 IN VITRO PHOSPHORYLATION AND GEL SHIFT

Purified MBP-A1 (*Maltose Binding Protein*) or MBP-MS2 (as control) was phosphorylated in vitro with the catalytic subunit of PKA (sigma). For kinase assays, 20 μ M MBP-A1 or MBP-MS2 was incubated with increasing amount of PKA (1, 2, 4 units) at 30 °C for 30min, in a buffer containing 50 mM Tris pH7.5, 5 mM MgCl₂, 0.1 mM ³²P-ATP in a final volume of 10 μ l. After the reaction, 2 μ l of 5x SDS loading buffer were added to stop the reaction, followed by 10 minutes of boiling. Phosphorylated proteins and free ³²P-ATP were then separated on 12% SDS PAGE gel. Gels were dried under vacuum and visualized with a BAS-2500 phosphoimaging system (Fujifilm) and ImageGauge software.

To perform the gel shift experiments, 20 μ M MBP-A1 or MBP-MS2 was first phosphorylated by incubating with 2 units of PKA at 30 °C for 30 minutes, in a buffer containing 50 mM Tris pH7.5, 5 mM MgCl₂, 0.1 mM ATP. Phosphorylated MBP-A1 was then serially diluted and used for complex formation with A1 winner RNA (UAGGGACUUAGGGUUGUAGGGA) in a buffer containing 50 mM Tris pH7.5, 0.4 μ M TAMK labeled A1 winner, 100 mM DTT, 0.1 mg/ml tRNA. Complex was formed at 30 °C for 20 minutes. Complex and free RNA were separated on 8% PAGE native gel for 2.5-3 hours at 250V. Native gels were pre-run under 250v for 1 hour before loading samples. Images were taken on Fujifilm FLA-5100 imaging system and processed with ImageGauge.

For competition assay, 0.4 μ M phosphorylated MBP-A1 was incubated with 0.4 μ M TAMK labeled A1 winner to form complex. Increasing amount of cold A1 winner RNA (self) or non-specific RNA (0 μ M, 0.2 μ M, 0.4 μ M, 0.8 μ M and 1.6 μ M) was supplied in the incubation buffer. Complex was formed at 30 °C for 20 minutes. Complexes and free RNA were separated

by native PAGE. Image was taken on Fujifilm FLA-5100 imaging system and processed with ImageGauge V4.0.

3.0 IDENTIFICATION OF MOLECULAR LINKS MEDIATING DEPOLARIZATION INDUCED C1 SPLICING SILENCING

3.1 INTRODUCTION

KCl induced depolarization changes C1 exon splicing pattern in neurons. However, the molecular links mediating the signal transduction have not been identified. In this section, I have investigated the roles of calcium signaling, CaMK IV pathway, PKA pathway and splicing silencer hnRNP A1 in mediating the signal transduction in depolarization induced C1 exon splicing silencing.

3.2 Ca^{2+} SIGNALING AND C1 SPLICING

3.2.1 KCl-Induced Sustained Ca^{2+} Influx is Associated With C1 Splicing

Silencing

Depolarization in neuron cells can induce Ca^{2+} influx. To test whether C1 splicing is regulated by Ca^{2+} influx, I monitored the intracellular Ca^{2+} concentration in neurons during depolarization. Relative calcium concentrations were displayed as a ratio of F340/F380 (Santos, Pierrot et al. 2009). To verify that the calcium concentration changed during depolarization, cells were treated with 50 mM KCl to induce cell depolarization. Upon the treatment of KCl, the cellular calcium concentration increased rapidly, and then decayed to about 40% of the maximal response (Figure 5A). Importantly, the elevated calcium levels were sustained. As a control, I used Ionomycin, a calcium ionophore, to induce calcium influx independent of depolarization. After a rapid influx, the cellular calcium concentration decayed to basal level, indicative of transient influx. Calcium levels were essentially identical at the midpoint (600sec) and at the end of experiment for KCl stimulated depolarization, whereas these levels decreased for Ionomycin treatment. In addition, treatment of cells with thapsigargin, which release the Ca^{2+} in ER, induced almost no influx compared with the treatment of KCl (Figure 5E). I next compared the splicing pattern of C1 cassette under these two conditions. Whereas the exon inclusion decreased from 82% to 50% for KCl stimulated samples, the splicing pattern remained unchanged in the presence of Ionomycin (Figure 5D). Thus the splicing silencing of C1 exon is associated with sustained increase of the intracellular calcium concentration as found under the depolarization conditions.

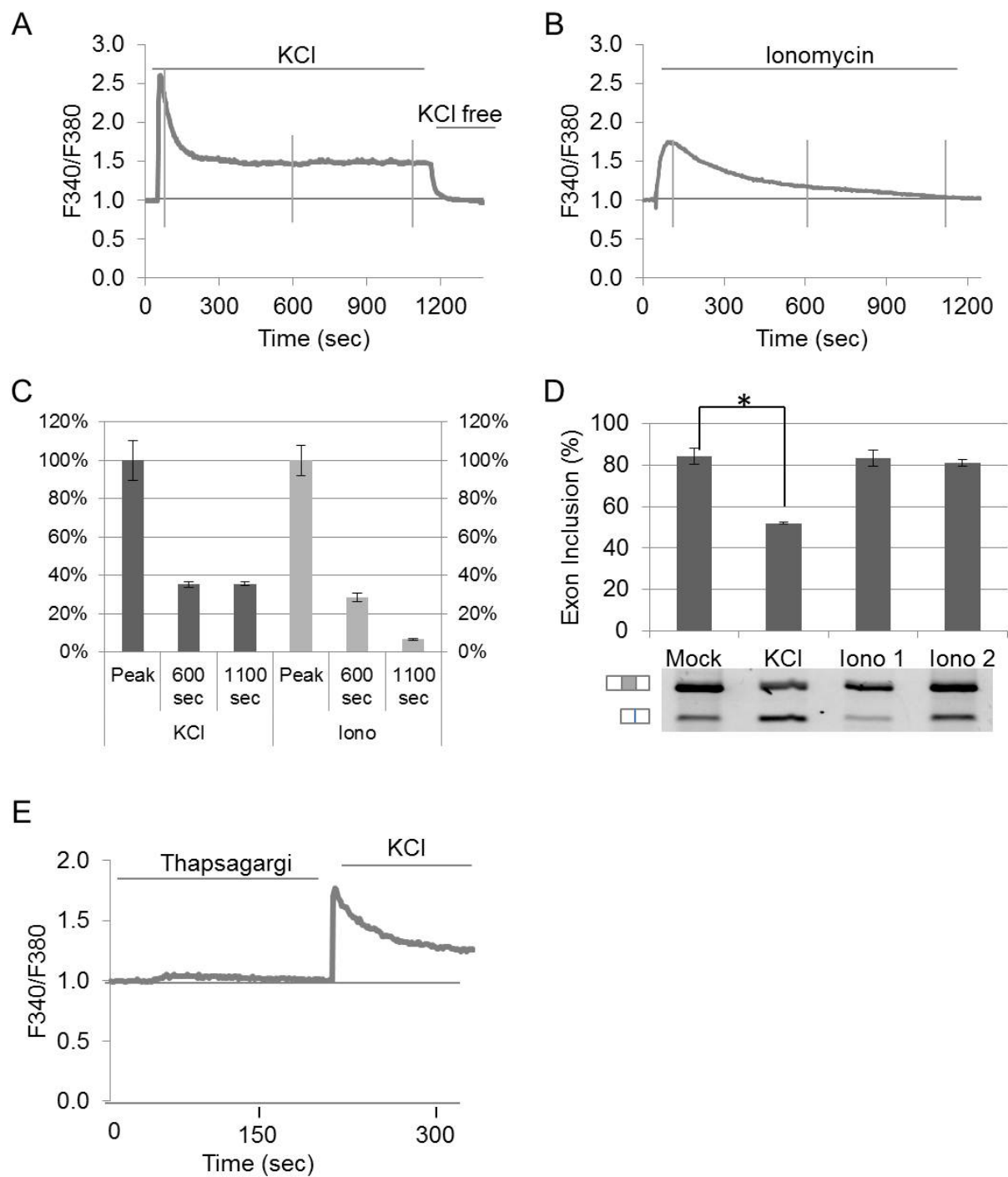


Figure 5. KCl induced C1 splicing silencing and sustained Ca^{2+} influx are associated

(A) High KCl induces sustained Ca^{2+} influx in cortical neurons. Ca^{2+} concentration in the cells was monitored by Ca^{2+} Imaging using 2 μM Fura-2AM. After about 20 minutes of KCl treatment to induce depolarization, KCl was washed out. (B) Ionomycin induces transient Ca^{2+} influx in cortical neurons.

Primary neurons were treated with 1 μ M Ionomycin. (C) Quantitation of Ca^{2+} concentration in cells. Ca^{2+} concentration at the peak, at the time points of 600 sec and 1100 sec (indicated in panel A and B with vertical lines) in both KCl and Ionomycin treated samples were quantitated and normalized to the peak respectively ($n \geq 3$). (D) Ionomycin has weaker silencing effect on C1 splicing, compared with high KCl. Primary neurons were mock treated or treated with 50 mM KCl, or Ionomycin (2 μ M for Iono1 and 6 μ M for Iono 2) for 8 hours before harvesting for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.0001$). (E) Neuronal cells were treated with 5 μ M Thapsagargi to release the Ca^{2+} from ER. As comparison, cells were treated with 50 mM KCl to induced depolarization after the treatment of Thapsagargi.

If C1 splicing silencing is induced by sustained Ca^{2+} influx, inhibition of KCl induced sustained Ca^{2+} influx should attenuate KCl induced C1 splicing silencing. To test this, I used H89 to inhibit the KCl induced Ca^{2+} influx. It has been reported that depolarization induced Ca^{2+} influx requires the activity of the PKA pathway (Skeberdis, Chevaleyre et al. 2006). Application of H89 effectively inhibited depolarization induced Ca^{2+} influx and returned the sustained Ca^{2+} influx almost to background level (Figure 6A). Pre-incubating cells with H89 resulted in the abolishment of depolarization induced Ca^{2+} influx (Figure 6C). To test whether inhibition of Ca^{2+} influx will block the depolarization induced C1 splicing silencing, H89 was added to media 1 hour before KCl treatment. As shown in Figure 6D, addition of H89 effectively blocked KCl induced C1 splicing silencing, indicating the requirement of Ca^{2+} influx for depolarization induced C1 skipping. This conclusion was further confirmed with NMDA receptor antagonist APV and MK801. Application of APV and MK801, which blocked Ca^{2+} influx (Figure 6B), inhibited KCl induced C1 splicing (An and Grabowski 2007).

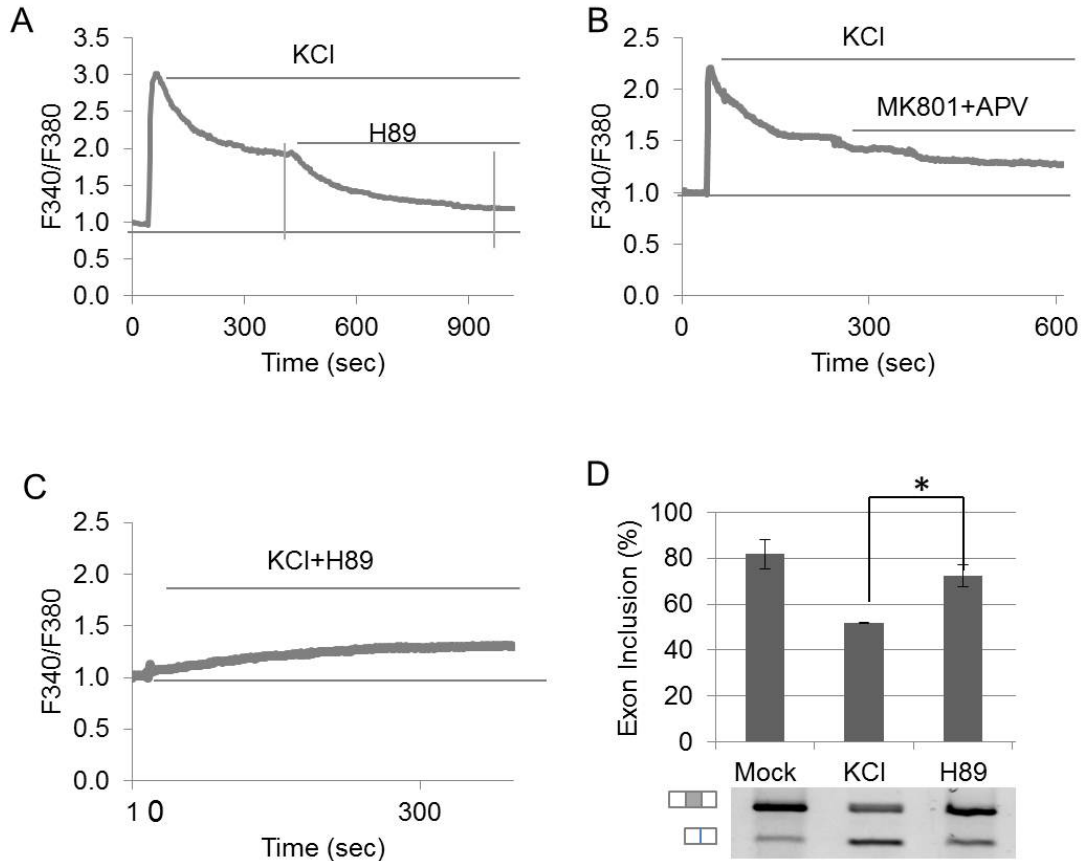


Figure 6. Inhibition of Ca^{2+} influx attenuates KCl induced C1 splicing silencing

(A) H89 inhibits KCl induced Ca^{2+} influx. Ca^{2+} concentration in neuron cells was monitored by Ca^{2+} imaging. Cells were first treated with 50 mM KCl to induce Ca^{2+} influx. After Ca^{2+} concentration reached plateau, 10 μM H89 was added into the buffer. (B) MK801 and APV inhibit KCl induced Ca^{2+} influx. Neuron cells were first treated with 50 mM KCl to induce Ca^{2+} influx. After Ca^{2+} concentration reached plateau, MK801 and APV were added into the buffer. (C) Pre-incubation of cells with H89 blocked Ca^{2+} influx. Cells were first incubated with 10 μM H89 for 10 minutes before Ca^{2+} imaging. Cells were treated with 50 mM KCl with 10 μM H89 to induced Ca^{2+} influx. (D) H89 attenuates KCl induced C1 splicing silencing. Primary neurons were mock treated or treated with 50 mM KCl for 8 hours before being harvested for RNA isolation and RT-PCR. For H89 treatment, cells were first treated with 10 μM H89 for 1 hour, then treated with 50 mM KCl. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.0039$).

To further test the roles of sustained Ca^{2+} influx in mediating C1 splicing skipping, I over-expressed NR1 and NR2 subunits of the NMDA receptor in N18TG2 cells. In this system, calcium influx is initiated by the application of NMDA receptor agonist glutamate and glycine, and is inhibited by the antagonist MK801 (Clarke and Johnson 2008). EYFP was used as a mark for transfected cells. Immediately after the addition of glutamine and glycine, intracellular calcium levels increased (Figure 7A). Importantly, addition of antagonist, MK801, returned calcium levels to the background. In the cells that were not transfected, no calcium level changes were detected. Similar to that observed in primary neurons, the increased calcium levels were sustained, although the maximal response was lower. Taking advantage of this system, I studied the splicing pattern of C1 splicing reporter in the NR1/NR2 over-expressed cells. In agreement with the requirement for intact NMDA receptor, C1 exon inclusion was reduced when co-transfected with NR1 and NR2 subunits, but not for co-transfected cells with individual subunit (Figure 7D, lane 1-3). This is what should be expected, if the splicing pattern is modulated by calcium influx through the NMDA receptor. In agreement with this, the induced silencing was blocked by NMDA receptor antagonists, APV or MK801 (Figure 7D, lane 4, 5). When Ionomycin was used as control, the maximal response of the calcium influx was higher than that observed for the reconstituted NMDA receptors (Figure 7C). However, different from sustained calcium influx induced by the reconstituted NMDA receptors, the Ionomycin induced calcium influx quickly decayed to basal level. As a result, no splicing changes were detected in the cells treated with Ionomycin. Thus, sustained calcium influx is associated with KCl induced C1 splicing silencing.

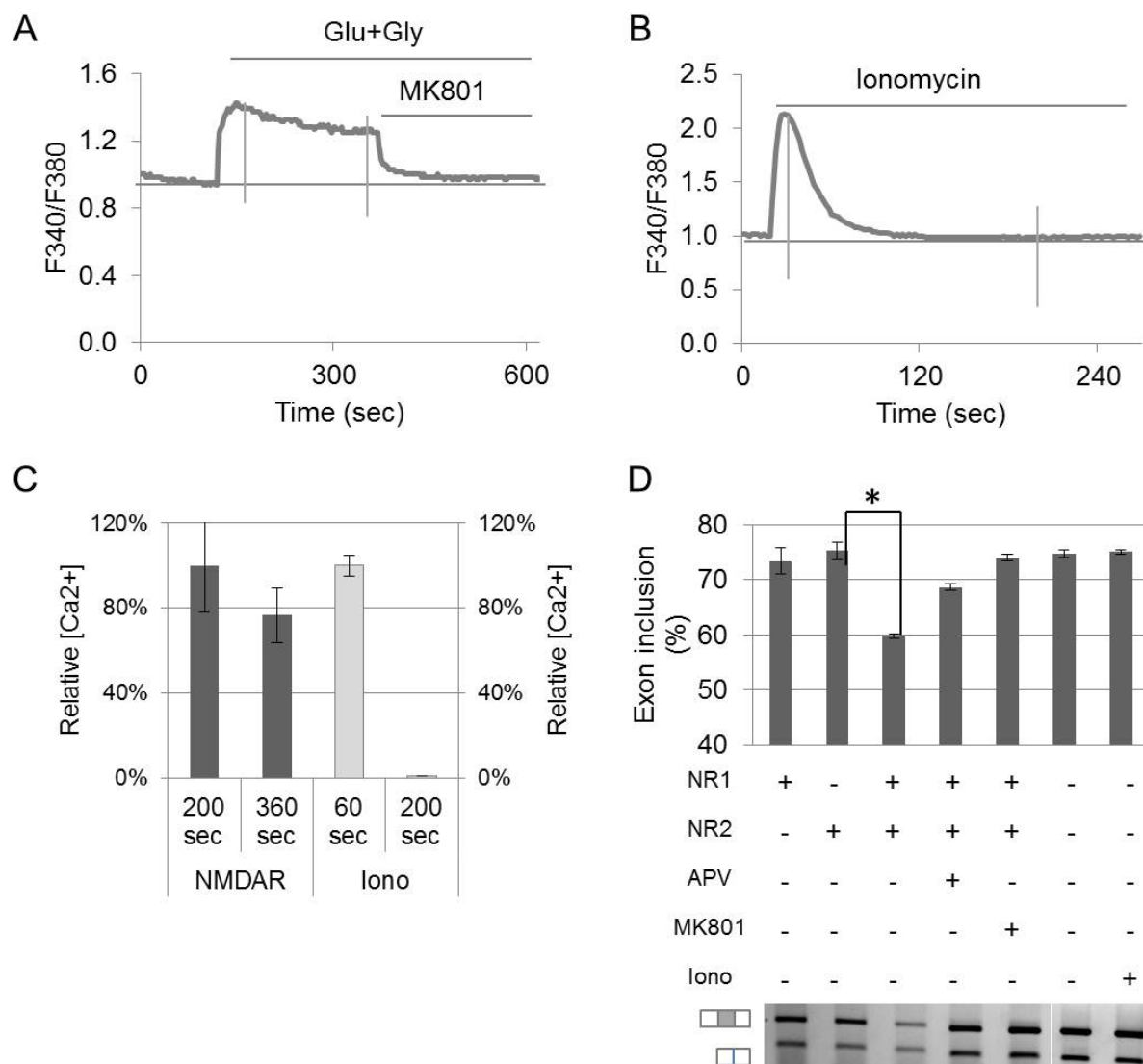


Figure 7. Induced sustained Ca²⁺ influx in N18TG2 cells promotes C1 splicing skipping

(A) Over-expression of NR1 and NR2 subunits of NMDA receptor in N18TG2 cells results in sustained Ca²⁺ influx when treated with Glycine and Glutamate. Ca²⁺ influx was monitored with Ca²⁺ imaging. Cells were transfected with NR1 and NR2 for 12 hours before Ca²⁺ imaging. An incubation buffer with 10 μ M Glycine and 30 μ M Glutamate was used to induce Ca²⁺ influx. After a plateau, the incubation buffer was washed out and changed into 10 μ M Glycine and 30 μ M Glutamate with 10 μ M MK801. (B) Ionomycin can only induce transient Ca²⁺ influx in N18TG2 cells. Cells were treated with 1 μ M Ionomycin to induce Ca²⁺ influx. (C) Quantitation of Ca²⁺ concentration in cells. Ca²⁺ concentration at the

peak and 360sec/200sec (indicated in panel A and B with vertical lines) in both KCl and Ionomycin treated samples were quantitated and normalized to the Ca^{2+} concentration at the peak, respectively ($n \geq 3$). (D) C1 splicing silencing was induced in N18TG2 cells over-expressing NR1 and NR2. Cells were transfected with NR1/NR2 subunits and C1 splicing reporter for 18-24 hours before being harvested for splicing assay. 200 μM APV or 10 μM MK801 were used to inhibit the NMDA receptor. For Ionomycin treatment, cells were first transfected with C1 splicing reporter for 5 hours. Then, media was changed and 2 μM Ionomycin was added for another 14 hours ($n \geq 3$, $p=0.0005$).

3.2.2 CaMK IV Induces C1 Splicing Silencing in a hnRNP A1 Dependent Manner

CaMK IV pathway is one of the main pathways that are directly activated by Ca^{2+} influx in neurons (Ghosh and Greenberg 1995; Redmond, Kashani et al. 2002). In addition, a few studies have implicated CaMK IV mediates depolarization induced C1 splicing silencing (An and Grabowski 2007; Lee, Xing et al. 2007). KCl induced C1 splicing silencing could be blocked by CaMK IV inhibitor KN93. And, over-expression of CaMK IV with C1 splicing reporter resulted in more C1 skipping. However, it is not clear which splicing factor is involved in the CaMK IV mediating C1 splicing silencing. hnRNP A1 (or A1 for convenience) is widely studied as a splicing silencer. Previously studies from our lab have shown hnRNP A1 is directly involved in KCl induced C1 splicing silencing (Han, Yeo et al. 2005; An and Grabowski 2007).

To determine if the CaMK IV mediated C1 splicing silencing is mediated by hnRNP A1, I knocked down hnRNP A1 in N18TG2 cells and asked if knockdown of A1 would attenuate

CaMK IV induced C1 splicing silencing. To do this, N18TG2 cells were first transfected with A1 siRNA. Knockdown of hnRNP A1 was verified by Western blot (Figure 8B). 24 hours after siRNA treatment, cells were transfected with constitutively active CaMK IV (ca CaMK IV) together with C1 splicing reporter for splicing assay. As shown in Figure 8A, in scrambled siRNA treated cells, over-expression of ca CaMK IV reduced C1 exon inclusion by 20% (from 80% to 60%). However, in the A1 siRNA treated cells, over-expression of ca CaMK IV had much less effect (less than 5%) on C1 exon inclusion, indicating the requirement of hnRNP A1.

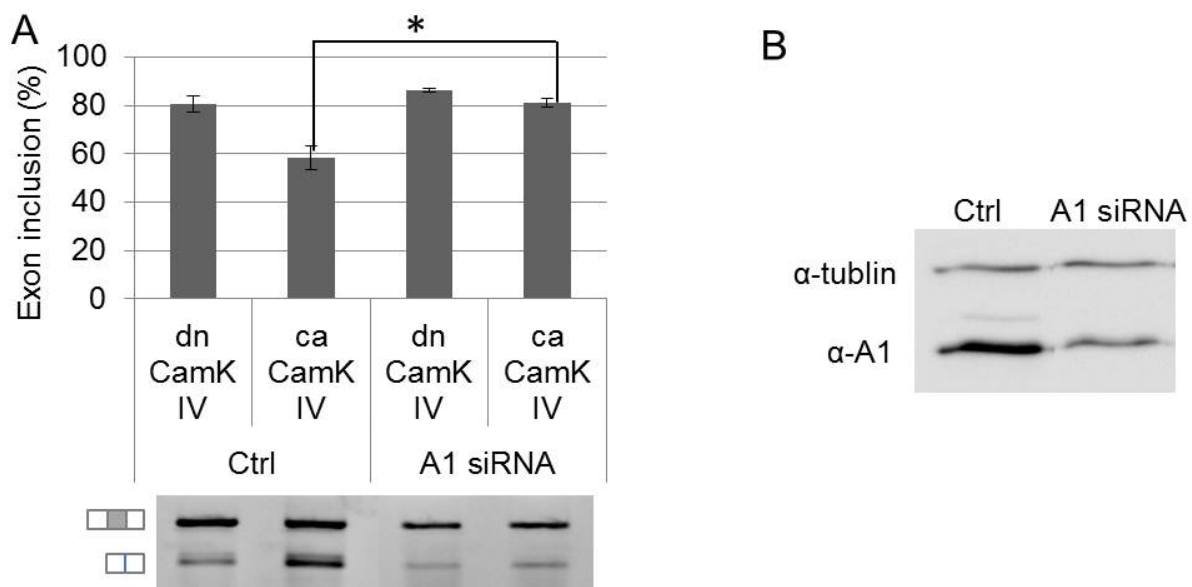


Figure 8. Knockdown of hnRNP A1 attenuates CaMK IV induced C1 splicing silencing

(A) N18TG2 cells were first treated with scrambled siRNA or A1 siRNA for 24 hours, and then transfected with CaMK IV and C1 splicing reporter for another 24 hours. Transfected cells were harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.002$). (B) N18TG2 cells were transfected with scrambled or A1 siRNA for 48 hours. Transfected

cells were harvested, and whole cells lysates were used for Western Blot against β -tubulin and A1 (4B10) as indicated in the figure. 70% hnRNP A1 was knocked down, as quantitated using Image Gauge 4.0.

Previously, our lab has shown that the UAGG motif in the C1 exon is critical for hnRNP A1 induced C1 splicing silencing (Han, Yeo et al. 2005). To further validate the dependence of hnRNP A1 in the CaMK IV induced C1 splicing silencing, I took advantage of the UAGG mutants. The structures of C1 wild type splicing reporter and UAGG mutants splicing reporter are shown in Figure 9A. In the C1 wild type, there are 2 UAGGs. When C1 wild type splicing reporter was over-expressed with hnRNP A1, C1 exon inclusion was reduced more than 20%. However, in the UAGG mutants (E8, E13 and E17), A1 induced C1 exon skipping was largely blocked. This suggests that UAGG motif is required for hnRNP A1 induced silencing. If the CaMK IV induced C1 splicing silencing is hnRNP A1 dependent, then the UAGG motif should also be required. To test this, ca CaMK IV was over-expressed with either the C1 wild type splicing reporter or the UAGG mutant splicing reporter. As expected, ca CaMK IV induced exon skipping was largely blocked in E8 and E13, and was completely blocked in E17. These data suggest that UAGG motif, which is required for hnRNP A1 induced C1 splicing silencing, is also required for CaMK IV induced C1 splicing silencing, consistent with the result that CaMK IV induced C1 splicing silencing is hnRNP A1 dependent.

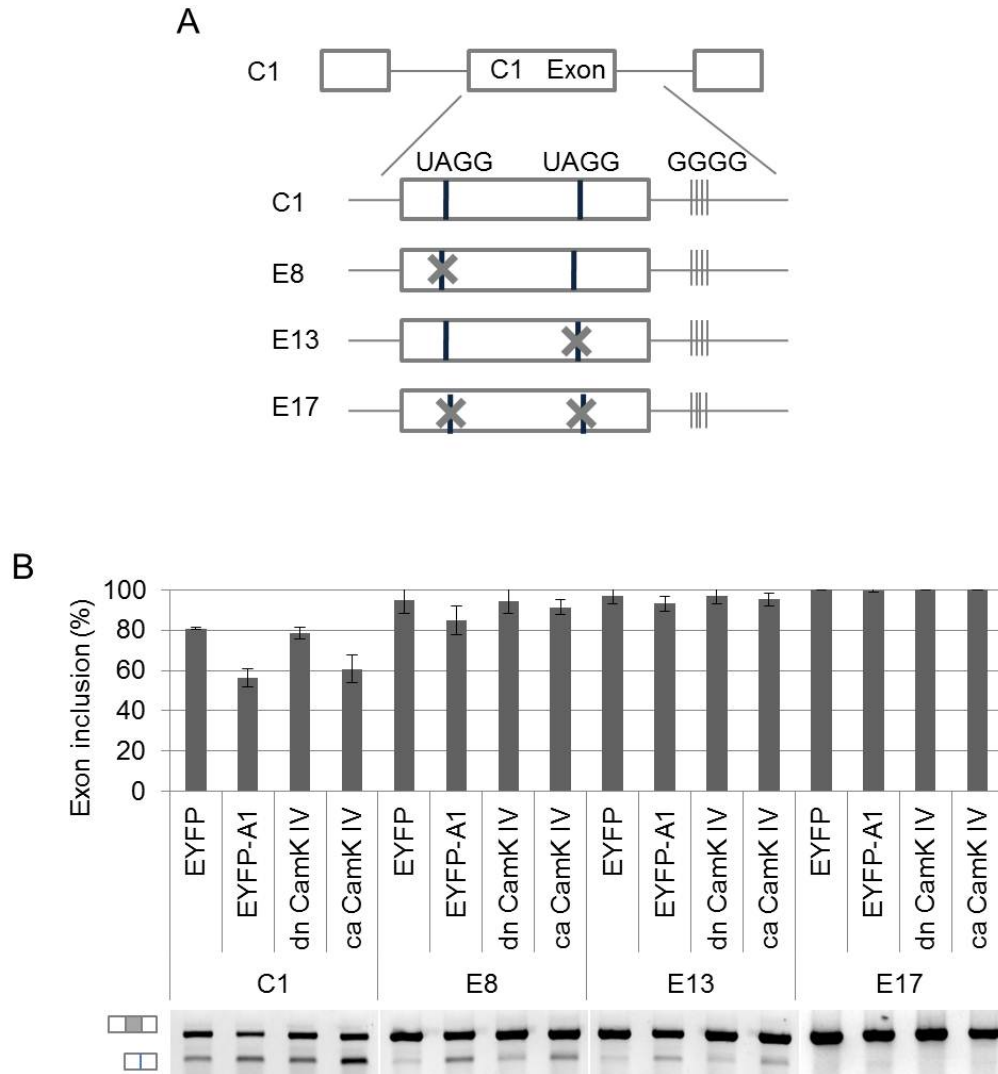


Figure 9. UAGG motifs in C1 exon are required for CaMK IV induced C1 splicing silencing

(A) Schematic structure of C1 splicing reporter and its UAGG mutants. C1 exon contains 2 UAGGs. The first UAGG is mutated in the splicing reporter E8, and the second UAGG is mutated in E13. E17 is the double mutant with no UAGG in the exon. (B) C1 splicing reporter and UAGG mutants (E8, E13 and E17) were transfected with EYFP, EYFP-A1, dn CaMK IV or ca CaMK IV for 24 hours. Transfected cells were then harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel.

3.3 PKA PATHWAY AND C1 SPLICING

3.3.1 CaMK IV Induces C1 Splicing Silencing in a hnRNP A1 Dependent Manner

PKA pathway is another main pathway activated by calcium influx in neurons (Ghosh and Greenberg 1995; Redmond, Kashani et al. 2002). To investigate the roles of PKA in regulating C1 splicing, I over-expressed constitutively active PKA (ca PKA) in N18TG2 cells with C1 splicing reporter. Relative to the transfection controls, over-expression of ca PKA promoted significant C1 splicing skipping (Figure 10A, lane 1, 3). However, over-expression of dominant negative PKA (dn PKA) had no effect on C1 splicing (Figure 10A, lane 1, 2). Interestingly, over-expression of ca PKA even had better silencing effect than ca CaMK IV.

I next wanted to see whether PKA could also induce C1 splicing silencing in a hnRNP A1 dependent manner. I took advantage of the hnRNP A1 knockdown system. To test this hypothesis, N18TG2 cells were first transfected with hnRNP A1 siRNA to knock down hnRNP A1, and then transfected with ca PKA and C1 splicing reporter. Similar to CaMK IV, in the scrambled siRNA treated cells, over-expression of ca PKA reduced more than 20% exon inclusion; while in the hnRNP A1 siRNA treated cells, over-expression of ca PKA reduced only about 5% exon inclusion. These data suggest that hnRNP A1 is also required for PKA induced C1 splicing silencing. To further validate this result, I took advantage of the UAGG mutants as I did for CaMK IV. As expected, both UAGGs, which were required for hnRNP A1 induced C1

splicing silencing, were also required for PKA induced C1 splicing silencing, further validating PKA mediated C1 splicing silencing requires hnRNP A1.

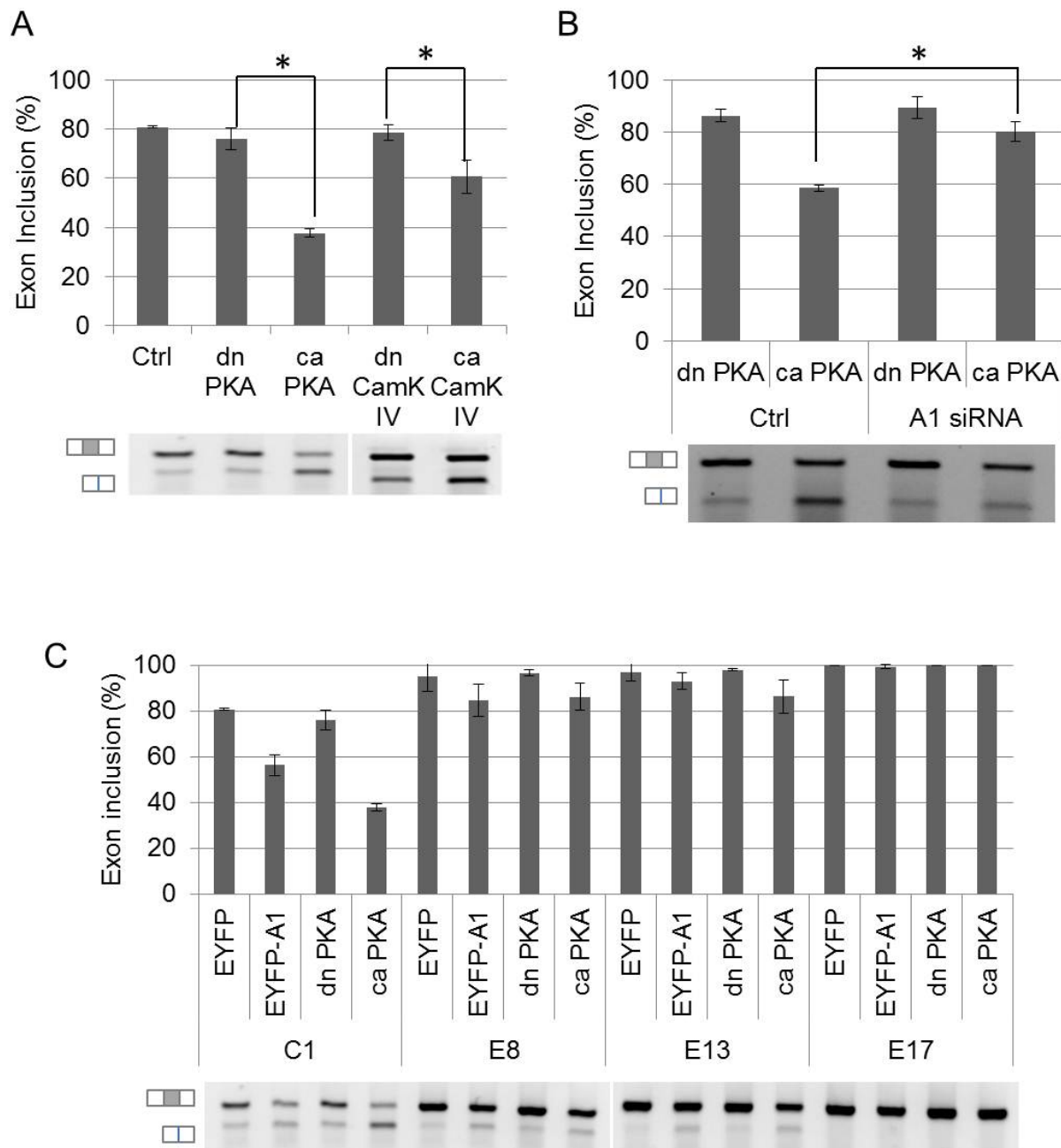


Figure 10. PKA regulates C1 splicing in a hnRNP A1 dependent manner

(A) Over-expression of active PKA in N18TG2 cells promotes C1 splicing silencing. N18TG2 cells were transfected with C1 splicing reporter and dn PKK/ca PKA for 18-24 hours before being harvested for

splicing assay. dn CaMK IV and ca CaMK IV were also transfected with C1 splicing reporter for comparison ($n \geq 3$, $p < 0.001$). (B) PKA silencing effect on C1 splicing is hnRNP A1 dependent. N18TG2 cells were first treated with scrambled siRNA or A1 siRNA for 24 hours, and then transfected with PKA and C1 splicing reporter for another 24 hours. Transfected cells were harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p = 0.0002$). (C) C1 splicing reporter and UAGG mutants (E8, E13 and E17) were transfected with EYFP, EYFP-A1, dn PKA or ca PKA for 24 hours. Transfected cells were then harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel.

3.3.2 Phosphorylation of hnRNP A1 Changes its RNA Binding Affinity

PKA regulates C1 splicing via splicing factor hnRNP A1, indicating that hnRNP A1 activity might be regulated by PKA. It has been reported that PKA can phosphorylate hnRNP A1 at site S199 in vitro (Cobianchi, Calvio et al. 1993). To verify that that PKA catalytic subunit can phosphorylate recombinant hnRNP A1 (MBP-A1) in vitro, I did a kinase assay with PKA. To find the best phosphorylation condition, increasing amount of PKA was used for phosphorylation (Figure 11A). As a MBP control, MBP-MS2 was also used in the kinase assay. As shown in Figure 11B, 0.5 unit of PKA was sufficient to phosphorylate 20 μ M MBP-A1 in 30 minutes. In the MBP-MS2 control, there was also some minor phosphorylation, indicating there might be some minor PKA phosphorylation sites in the MBP or MS2. However, the phosphorylation was weak.

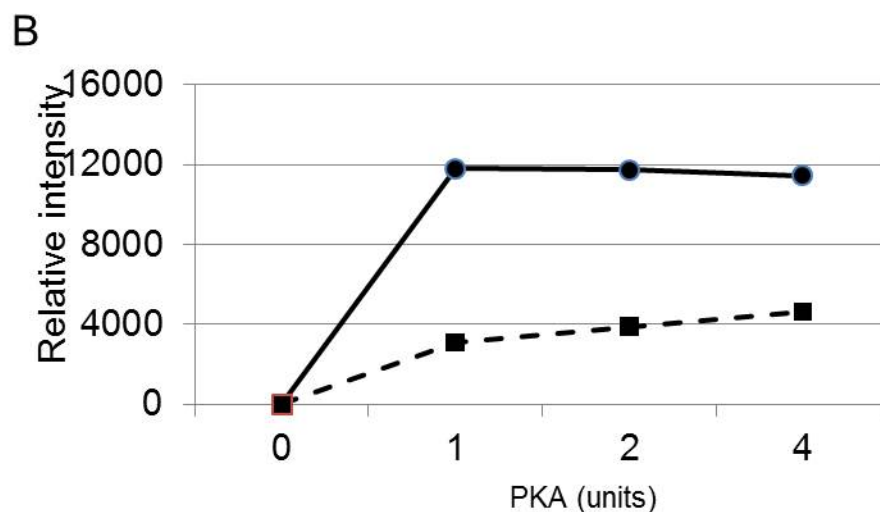
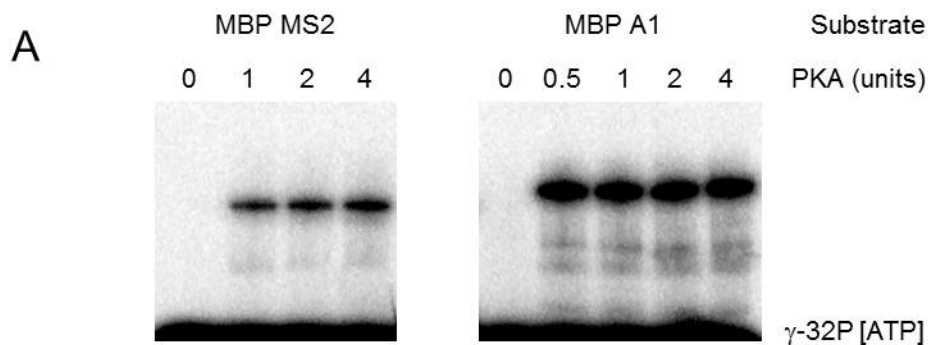


Figure 11. PKA phosphorylates MBP-A1 in vitro

(A) MBP-A1 or MBP-MS2 (as a control) was treated with PKA at 30°C for 30 minutes. Amounts of PKA for phosphorylation are indicated in the figure. After phosphorylation, MBP-A1 and free ³²P-ATP were separated by SDS-PAGE. Gels were dried under vacuum and visualized with a BAS-2500 phosphoimaging system (Fujifilm) and ImageGauge software. (B) Quantitation of phosphorylated MBP-A1 or MBP-MS2. Bands were quantitated with ImageGauge software. Solid line and dots stand for the intensity of phosphorylated MBP-A1 with the treatment of various amounts of PKA. Dotted line and squares stand for the intensity of phosphorylated MBP-MS2 with the treatment of various amounts of PKA.

To test if the phosphorylation changed hnRNP A1 RNA binding affinity, I did a gel shift with purified MBP-A1 and TAMK labeled RNA oligo A1 winner (UAGGGACUUAGGGUUGUAGGGA), which is known bind to hnRNP A1. PKA or mock treated MBP-A1 was used for complex formation with A1 winner. Complex(es) and free RNA were then separated on native PAGE gel. As shown in Figure 12A, more complex were formed in samples treated with PKA, suggesting that phosphorylation increases MBP-A1 RNA binding affinity. Interestingly, more complex II was found in the PKA treated samples, indicating phosphorylation might promote MBP-A1 dimerization. To rule out that MBP might contribute to the complex formation with A1 winner, MBP-MS2 was used as control for complex formation. However, no complex was detected in either PKA or mock treated samples (Figure 12B).

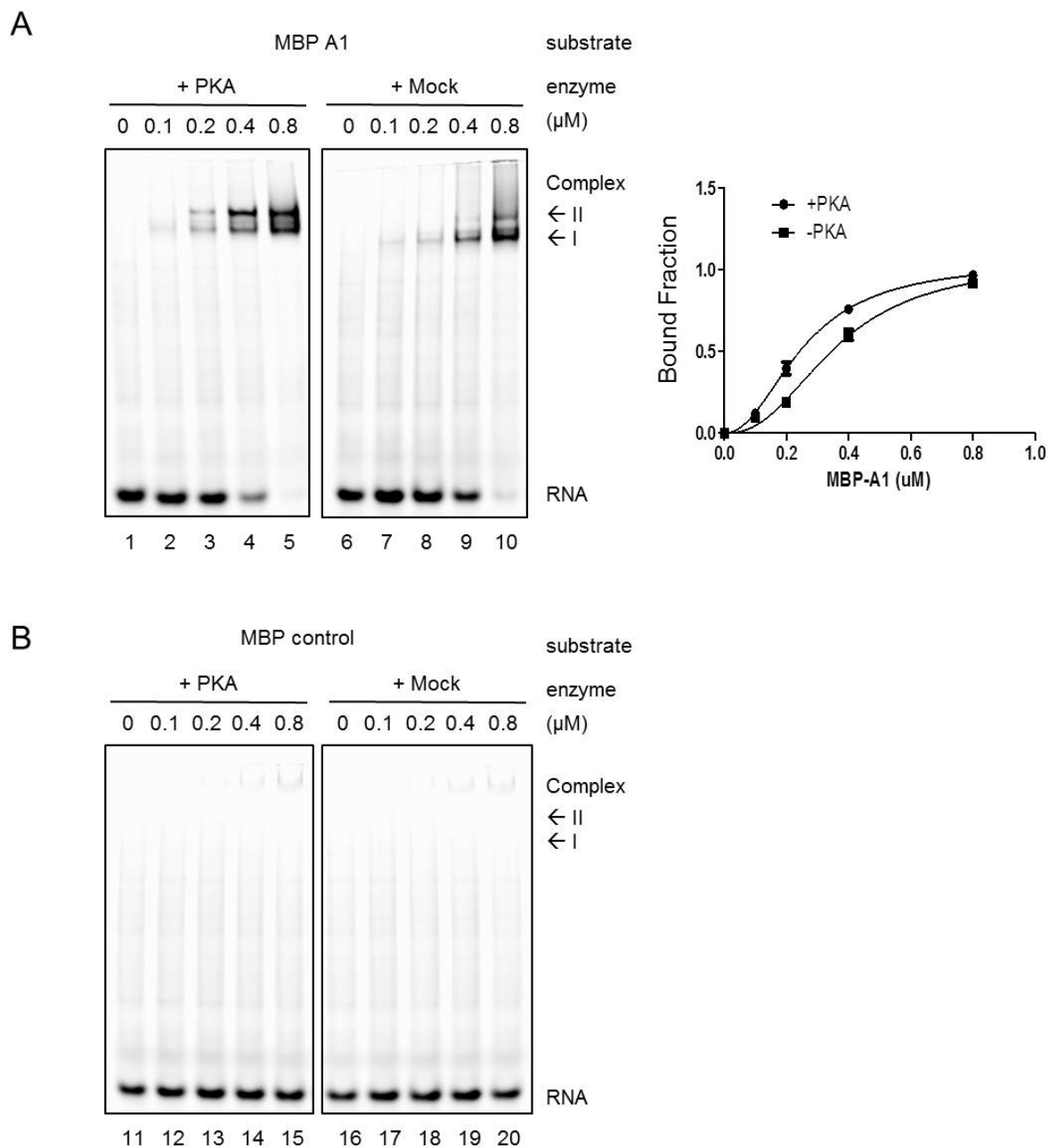


Figure 12. Phosphorylation of hnRNP A1 by PKA increases its RNA binding affinity

(A) MBP-A1 was either treated with PKA or mock treated, and then incubated with 0.4 μ M TAMK labeled A1 winner for complex formation. Increasing amount of MBP-A1 was used for complex formation, as indicated in the figure. Complexes and free RNA were separated on 8% native PAGE gel. Image was taken on Fujifilm FLA-5100 imaging system and processed with image gauge V4.0. Complex I (A1 monomer) and Complex II (A1 dimer) are labeled. The quantitation results from at least three

experiments are shown on the left. Data were fit with Graphpad. (B) MBP control MBP-MS2 was either treated with PKA or mock treated, and then incubated with 0.4 μ M TAMK labeled A1 winner for complex formation. Amount of MBP-MS2 used for complex formation is indicated in the figure. Complexes and free RNA were separated on native PAGE gel. Image was taken on Fujifilm FLA-5100 imaging system and processed with ImageGauge V4.0. No complex was detected.

To show that MBP-A1 and A1 winner (RNA) binding is specific, cold A1 winner or non-specific RNA was used for competition experiments. With increasing concentration of cold A1 winner (non-labeled A1 winner), less complex was detected (Figure 13A, lane 1-5 and Figure 13B, lane 1-5). However, in the competition experiments with non-specific RNA, complexes formation was not affected (Figure 13A, lane 6-10 and Figure 13B, lane 6-10), indicating MBP-A1 and A1 winner binding is specific.

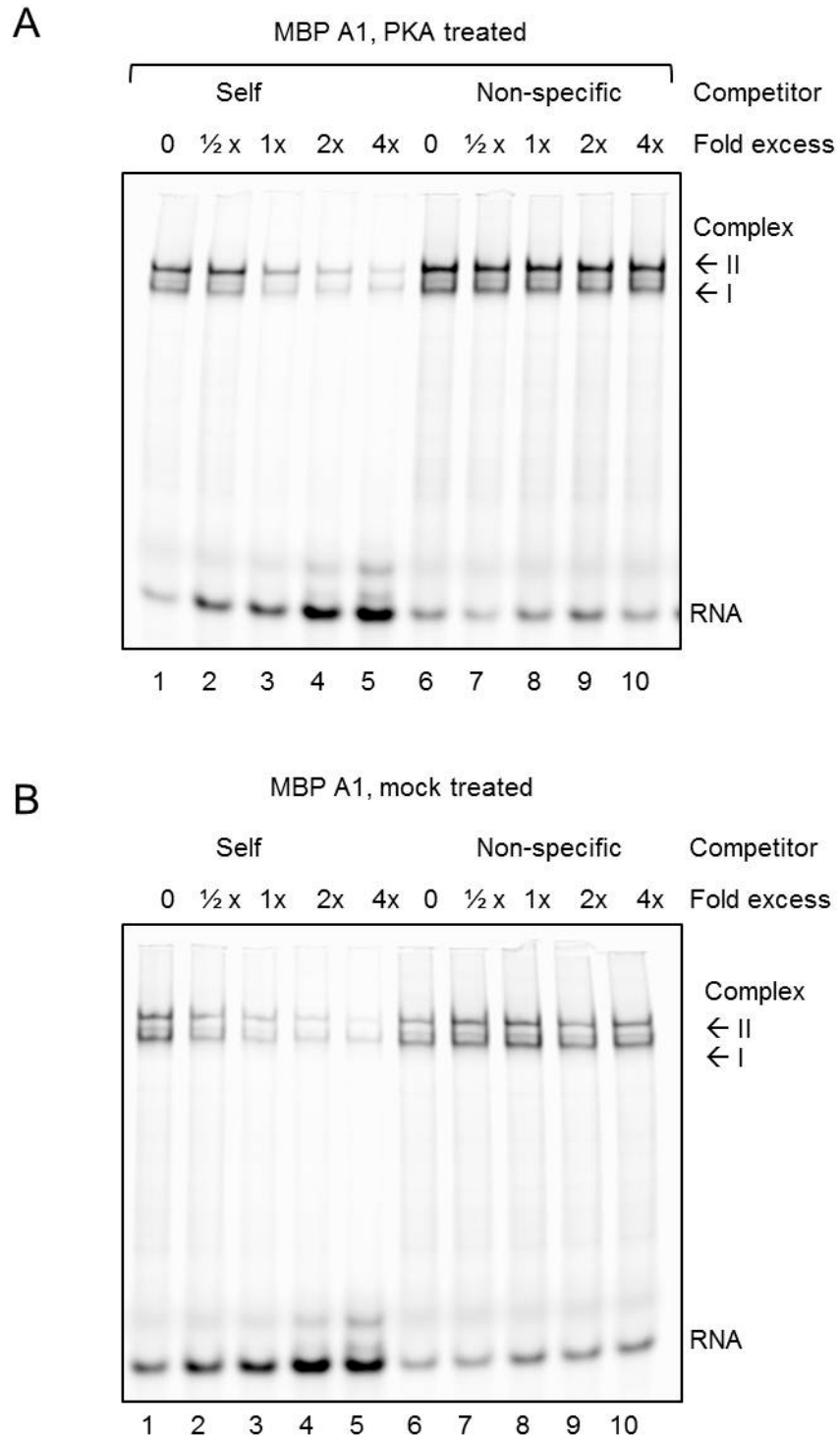


Figure 13. MBP-A1 binding to A1 winner is specific

(A) Competition experiment for PKA treated MBP A1. 0.4 μ M phosphorylated MBP-A1 was incubated with 0.4 μ M TAMK labeled A1 winner to form complex. Cold A1 winner RNA (self) or non-specific

RNA was supplied in the incubation buffer at the concentration indicated in the figure for competition. Complexes and free RNA were separated on 8% native PAGE gel. Image was taken on Fujifilm FLA-5100 imaging system and processed with ImageGauge V4.0. Complex I (A1 monomer) and Complex II (A1 dimer) are labeled. (B) Competition experiment for mock treated MBP A1. 0.4 μ M phosphorylated MBP-MS2 was incubated with 0.4 μ M TAMK labeled A1 winner to form complex. Cold A1 winner RNA (self) or non-specific RNA was supplied in the incubation buffer at the concentration indicated in the figure for competition. Complexes and free RNA were separated on native PAGE gel. Image was taken on Fujifilm FLA-5100 imaging system and processed with ImageGauge V4.0. Complex I (A1 monomer) and Complex II (A1 dimer) are labeled.

3.3.3 hnRNP A1 is Phosphorylated by PKA in vivo

To further demonstrate that PKA changes hnRNP A1 activity by phosphorylation, I wanted to see whether PKA could phosphorylate hnRNP A1 in vivo. To test this, increasing amount of ca PKA was over-expressed in N18TG2 cells. dn PKA was used as a control. To detect if hnRNP A1 was phosphorylated at S199, whole cell lysates were used for Western blot with antibody α - A1 S199p, which recognizes phosphorylated S199. Although α -A1 S199p can also recognize un-phosphorylated hnRNP A1, it has higher affinity for phosphorylated hnRNP A1 (Figure 14D). Not surprisingly, double bands were detected with antibody α -A1 S199p. Quantitation of these bands showed that the ratio of top/ bottomband increased with increasing amount of ca PKA, indicating that over-expression of PKA increases the phosphorylation of S199 (Figure 14A). Consistent with this, splicing assay using C1 splicing reporter showed that more C1 splicing was repressed with increasing amount of ca PKA (Figure 14B). These results suggest that

phosphorylation of hnRNP A1 by PKA at site S199 increase its silencing activity. This conclusion was supported by the Western blot with α -A1 S199p from depolarized neuron cells. In the KCl treated neuronal cells, increasing amount of A1 phosphorylation was detected with α -A1 S199p as time lapsed (Figure 14C). This result also suggests that depolarization induced C1 splicing silencing might be mediated by the PKA pathway.

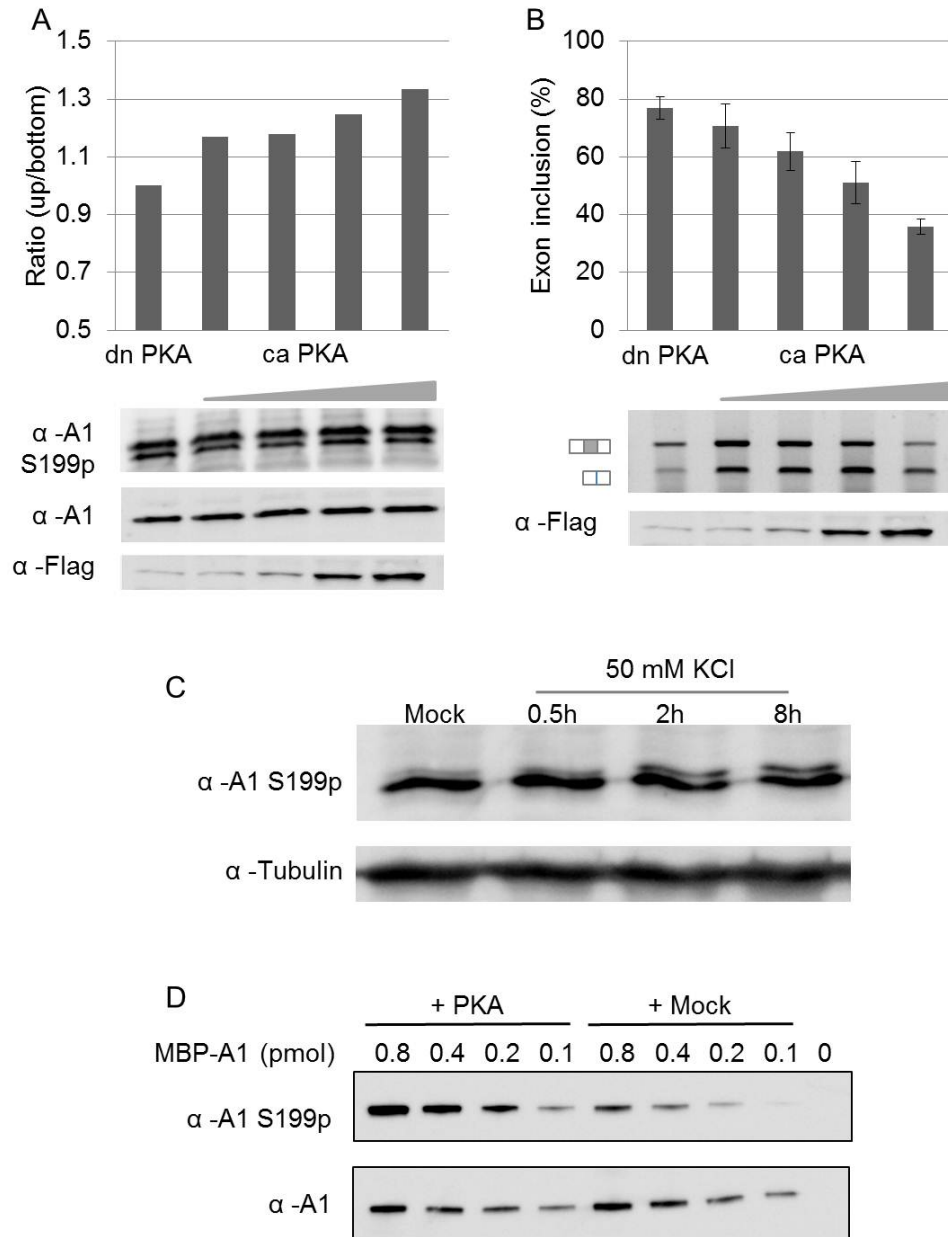


Figure 14. hnRNP A1 is phosphorylated by PKA in vivo

(A) Over-expression of ca PKA in N18TG2 cell phosphorylates hnRNP A1. N18TG2 cells were transfected with Flag tagged dn PKA (as control) or increasing amount of ca PKA. After 24 hours of transfection, cells were harvested and whole lysates were used for Western Blot with antibody α -A1 S199p, α -A1 and α -Flag. The α -A1 S199p bands were quantitated with ImageGauge, and the relative ratio of top/bottom band is shown in the figure. (B) N18TG2 cells were transfected with Flag tagged dn PKA (as control) or increasing amount of ca PKA. After 24 hours of transfection, cells were harvested for

RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel. (C) hnRNP A1 was phosphorylated after depolarization in primary neuron cells. Cells were mock treated or treated with KCl for the time indicated in the figure. Treated cells were then harvested and whole cell lysates were used Western Blot with antibody α -A1 S199p and α -tubulin (as loading control). (D) α -A1 S199p can recognize both phosphorylated and un-phosphorylated MBP-A1, but prefers phosphorylated MBP-A1. MBP-A1 was mock treated or treated with PKA. Various amounts of MBP-A1, as indicated in the figure, were used for Western blot with α -A1 S199p, α -A1 antibody.

3.3.4 CaMK IV Works Upstream of PKA

I have shown that both CaMK IV and PKA pathway regulate C1 splicing in a hnRNP A1 dependent manner. However, it is not clear about the relationship between CaMK IV and PKA pathway in regulating C1 splicing. To test this, I took advantage of the dominant negative CaMK IV and dominant negative PKA. If CaMK IV is upstream of PKA in the pathway, over-expression of dn PKA together with ca CaMK IV should be able to attenuate the CaMK IV induced splicing silencing, vice versa. dn CaMK IV was first over-expressed with ca PKA to see if dn CaMK IV could block PKA induced splicing silencing. To ensure there was a sufficient amount of dn CaMK IV, ca PKA and dn CaMK were transfected at a ratio of 1:2. However, as shown in Figure 15 (left), over-expression of dn CaMK IV had no effect on PKA induced C1 splicing silencing, indicating that PKA is not upstream of CaMK IV. Next, I over-expressed dn PKA with ca CaMK IV (2:1 ratio). Surprisingly, CaMK IV induced C1 splicing silencing was significantly attenuated by the over-expression dn PKA, indicating that CaMK IV acts upstream of PKA in regulating C1 splicing.

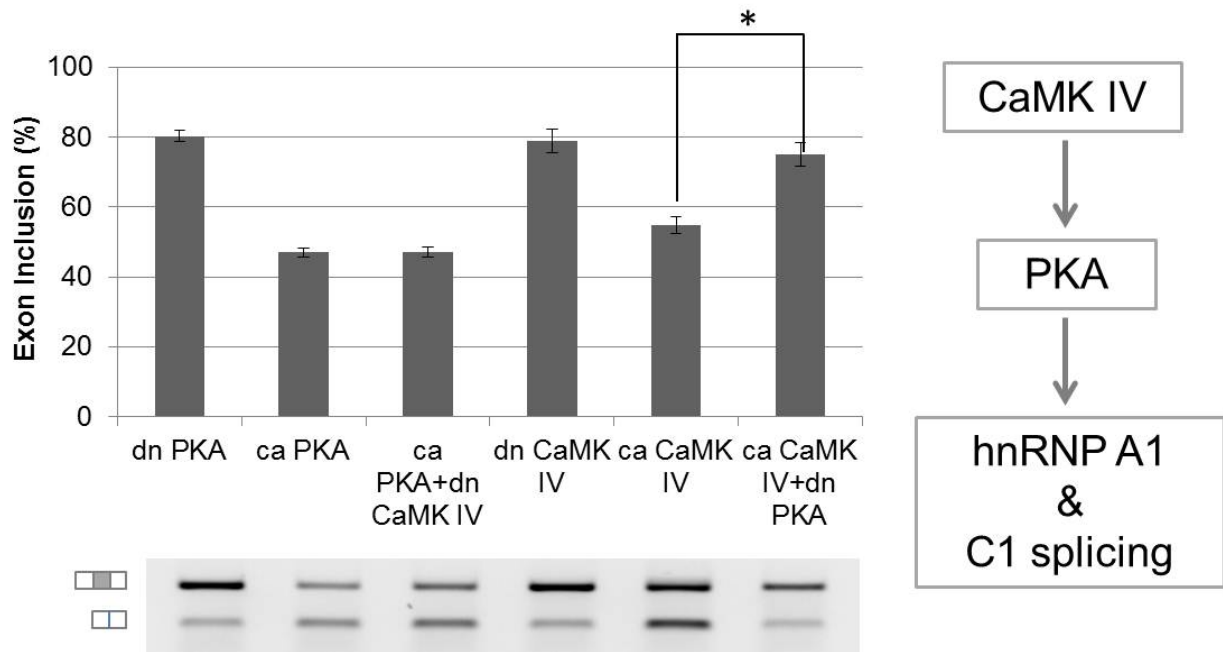


Figure 15. CaMK IV works upstream of PKA pathway

N18TG2 cells were transfected with PKA or CaMK IV as indicated in the figure. Transfected cells were harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.0011$). PKAm: dominant negative PKA. PKA: constitutively active PKA. DN: dominant negative CaMK IV. CA: constitutively active CaMK IV.

3.4 HNRNP A1 AND C1 SPLICING

3.4.1 C1 Splicing is Modulated by hnRNP A1

Previous work from our lab has shown that hnRNP A1 is involved in the regulation of C1 cassette splicing silencing in primary neurons (Han, Yeo et al. 2005; An and Grabowski 2007).

To further determine the roles of hnRNP A1 in regulating C1 splicing, hnRNP A1 was overexpressed in C2C12 cells with C1 splicing reporter. As shown in Figure 16C, in the transfected cells, over-expression of hnRNP A1 reduced the C1 exon inclusion in a dose dependent manner. When C1 reporter and hnRNP A1 were transfected at a ratio of 1:4, about a 40% decrease of C1 exon inclusion was observed. Previously, our lab has shown that KCl induced depolarization resulted in decrease of C1 exon inclusion in primary neurons (An and Grabowski 2007). To determine if over-expression of hnRNP A1 can also reduce C1 exon inclusion in primary neurons, hnRNP A1 was overexpressed in primary neurons with a C1 splicing reporter. As a control, primary neurons were also treated with KCl. As shown in the Figure 16D, over-expression of hnRNP A1 in neuron cells induced more than 20% decrease of C1 exon inclusion, consistent with the results from C2C12 cells. These results showed that overexpression of hnRNP A1 can silence the splicing of C1 cassette exon in both C2C12 cells and primary neurons.

I then ask if knockdown of hnRNP A1 in cells can increase C1 inclusion. Due to the low transfection efficiency in primary neurons, HeLa cells were used for knockdown. hnRNP A1 in HeLa cells was knocked down with A1 siRNA and then transfected with C1 wild type splicing reporter. Western blot showed that at least 50% A1 in the HeLa cells was knocked down in the cells transfected with A1 siRNA as compared to the mock control (Figure 16B). Splicing assay showed that C1 exon inclusion was almost doubled in the A1 knockdown cells (Figure 16E). Together with over-expression results, these data suggest that hnRNP A1 is a splicing silencer of C1 cassette exon.

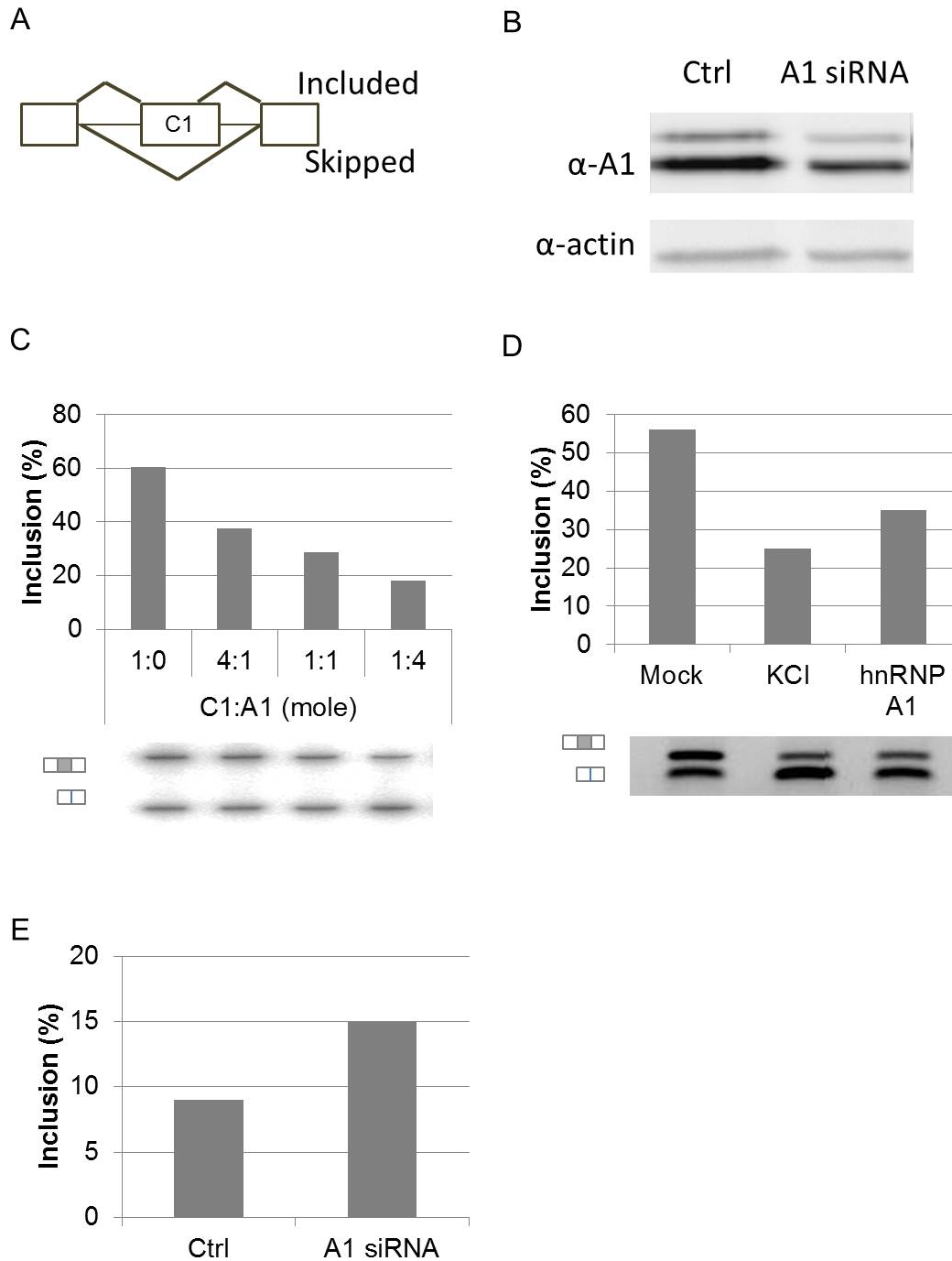


Figure 16. C1 splicing is modulated by hnRNP A1

(A) C1 exon can be either included or skipped. (B) C2C12 cells were transfected with scrambled or A1 siRNA for 48 hours. Transfected cells were harvested, and whole cells lysates were used for Western Blot against actin and A1 (4B10) antibody as indicated in the figure. (C) C1 splicing reporter was transfected

with increasing amount of hnRNP A1 into C2C12 cells. After 24 hours of transfection, cells were harvested for RNA isolation and RT-PCR. (D) C1 splicing reporter was transfected with hnRNP A1 into neurons for 24 hours before being harvested for the splicing assay. As a control, neuronal cells were either mock treated or treated with 50 mM KCl to induced depolarization. (E) C2C12 cells were first transfected with scrambled or A1 siRNA for 24 hours, and then transfected with C1 splicing reporter for another 24 hours for the splicing assay.

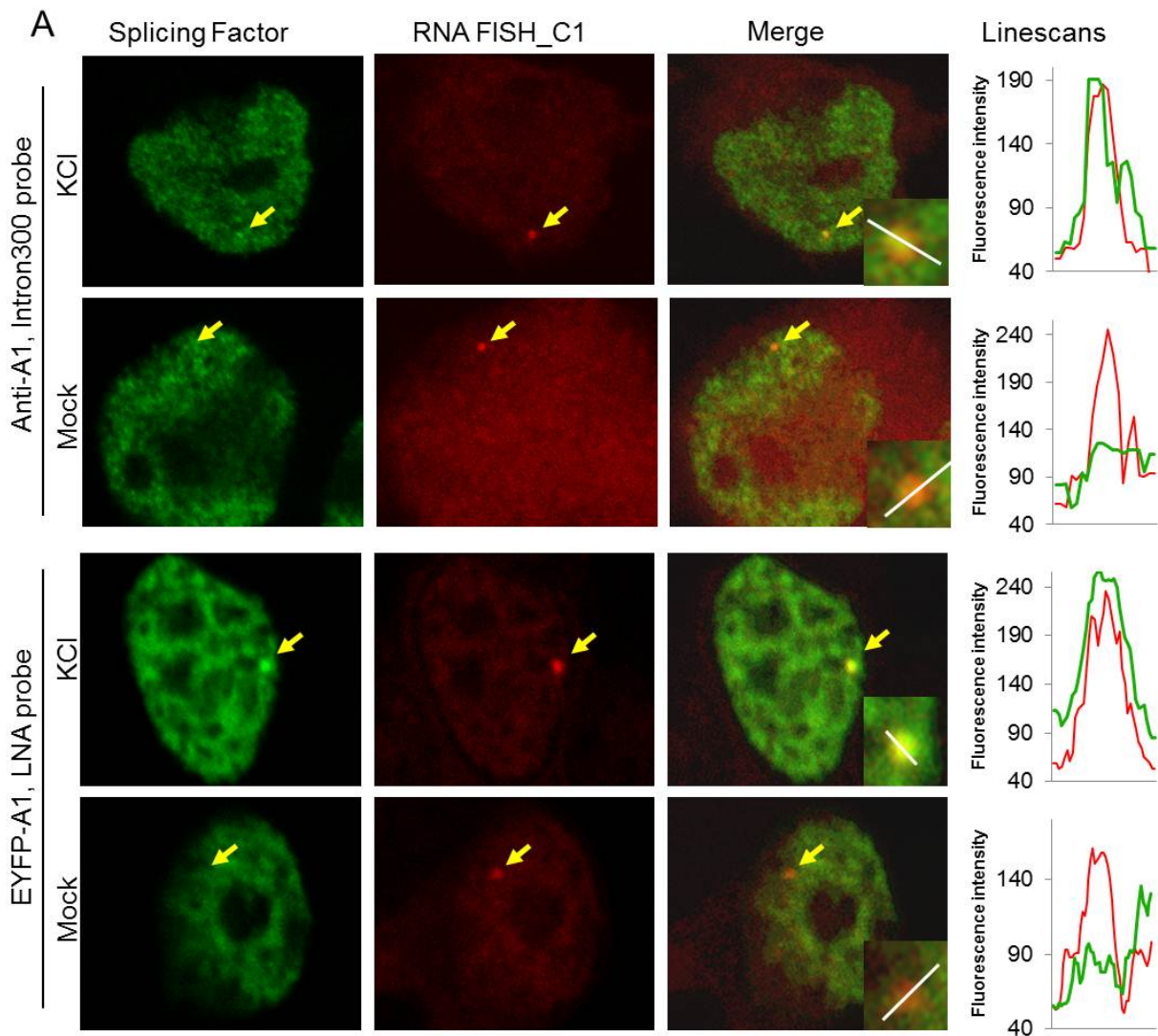
3.4.2 Depolarization Induces hnRNP A1 Recruitment

Using biochemical methods, our lab previously showed an increased binding of hnRNP A1 to the C1 UAGG motif in nuclear extract from KCl treated neurons (An and Grabowski 2007). However, it still remains unclear whether the regulation involves direct association of hnRNP A1 with C1 pre-mRNA transcript in vivo. To this end, I asked whether hnRNP A1 is recruited to C1 splicing sites in vivo after KCl induced depolarization. To determine the recruitment of hnRNP A1, I performed a quantitative single cell splicing factor recruitment assay which had been previously characterized by Tom Misteli and David L. Spector (Misteli, Caceres et al. 1998). In this assay, recruitment of endogenous hnRNP A1 to the transcription sites of C1 is detected by simultaneous RNA fluorescent in situ hybridization (FISH) to visualize the sites of C1 transcription using a DNA intron probe and indirect immunofluorescence (IF) using hnRNP A1 antibodies (9H10) to detect endogenous hnRNP A1. Positive recruitment is defined by two simultaneous criteria: a) the hnRNP A1 signal is accumulated at the transcription site more than

1.5-fold above the average hnRNP A1 nucleoplasmic signal, and b) at least half the hnRNP A1 intensity peak overlaps with at least half of the RNA-FISH intensity peak (Mabon and Misteli 2005). To perform the experiment, primary neurons were isolated and cultured in vitro for 8 days (DIV8) before being treated with 50mM KCl to induced cell depolarization. After 8 hours of KCl treatment, cells were fixed for RNA FISH and immune-staining. In the mock treated cells, hnRNP A1 was recruited to 39% of C1 splicing sites. However, the recruitment increased to 86% in the cells treated with KCl (Figure 18A, lane 1 and 2) (Sample images are shown in Figure 17). This suggests that treatment of cells with KCl induces more recruitment of hnRNP A1 to C1 splicing sites. To further validate this result, I performed another recruitment assay using recombinant hnRNP A1 (EYFP hnRNP A1) and a DNA/LNA (locked nucleic acid) probe which binds specifically to the exon/intron junction as tested by dot blot (data not shown). At DIV 7, primary neurons were transfected with EYFP A1 for 24 hours, followed by depolarization treatment. After treatment, cells were fixed for RNA FISH as described in the method (Chapter 2). In this assay, EYFP hnRNP A1 was recruited to 59% of C1 splicing sites in mock treated cells, while recruited to 90% in depolarized cells (Fig 18A, lane 3 and 4). The basal level of EYFP hnRNP A1 recruitment was higher than that of endogenous hnRNP A1 (59% vs 39%). This might be caused by the over-expression of hnRNP A1 in the cells. To this end, I have shown the recruitment hnRNP A1 (both endogenous and recombinant) increased more than 30% by cell depolarization. This result suggests that KCl induced cell depolarization induces hnRNP A1 recruitment to C1 transcription sites.

To show that the recruitment of hnRNP A1 to C1 exon was specific, I performed recruitment assays for other splicing factors. However, as shown in Figure 18B, none of these splicing factors (SC35 and ASF/SF2) showed significant recruitment to the transcription sites of

C1 under either resting or depolarization conditions. In addition, recruitment assay using MEN1 intron probe showed that recruitment of A1 to splicing sites of MEN1 was not changed by KCl treatment. Note that the splicing of MEN1 is not regulated by depolarization (Figure 18C), although it contains UAGG motif. These data indicate that KCl induced hnRNP A1 recruitment to the splice sites of C1 is specific.



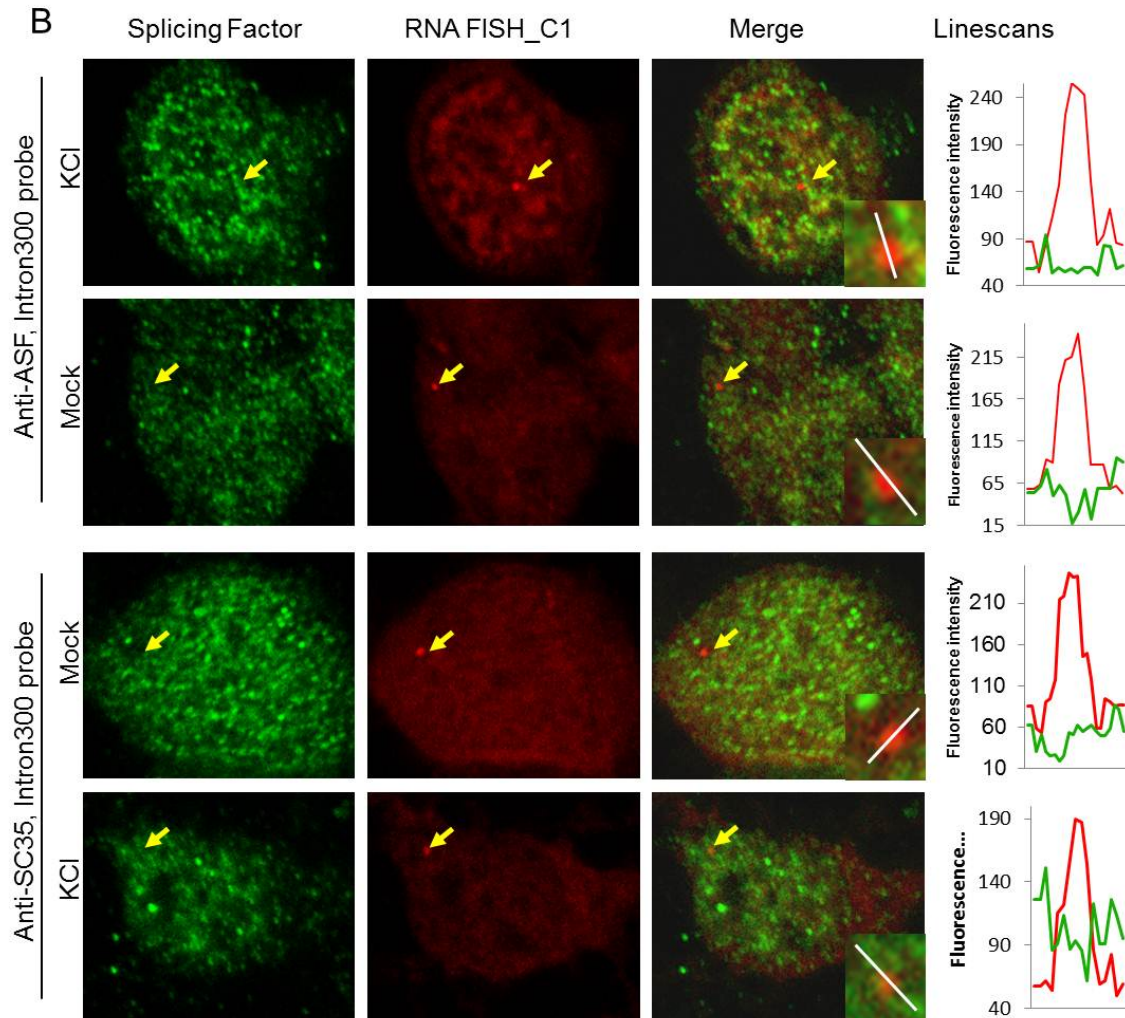


Figure 17. Recruitment assay of hnRNP A1 and other splicing factors

(A) Recruitment assay of endogenous and recombinant hnRNP A1. For endogenous A1, recruitment was detected by combined RNA-FISH using fluorescence labeled DNA probe (red) and IF microscopy with A1 antibody (9H10) (green). For recombinant A1, A1 was fused with EYFP (green), and transcription sites of C1 was detected with fluorescence labeled LNA probe (red). Arrows indicate C1 transcription sites. Insets are the higher magnifications of the transcription sites. All associations were confirmed with linescan analysis. Lines indicate the locations of the linescan. (B) Recruitment assay of ASF/SF2 and SC35. Recruitment was detected by combined RNA-FISH using fluorescence labeled DNA probe (red) and IF microscopy with ASF/SF2 or SC35 specific antibodies.

I have shown above that PKA is involved in the regulation of C1 splicing silencing in a hnRNP A1 dependent way. In addition, KCl treatment can also phosphorylate hnRNP A1 at S199. To this end, I want to test whether the activation of PKA pathway can stimulate hnRNP A1 recruitment. To do this, I used forskolin to activate the PKA pathway in the primary neurons, followed by recruitment assay. In the cells treated with 3 μ M forskolin, the recruitment of hnRNP A1 to C1 splicing sites was comparable with that in the cells treated with KCl (Figure 18D). This further confirms the roles of PKA in mediating KCl induced C1 splicing silencing.

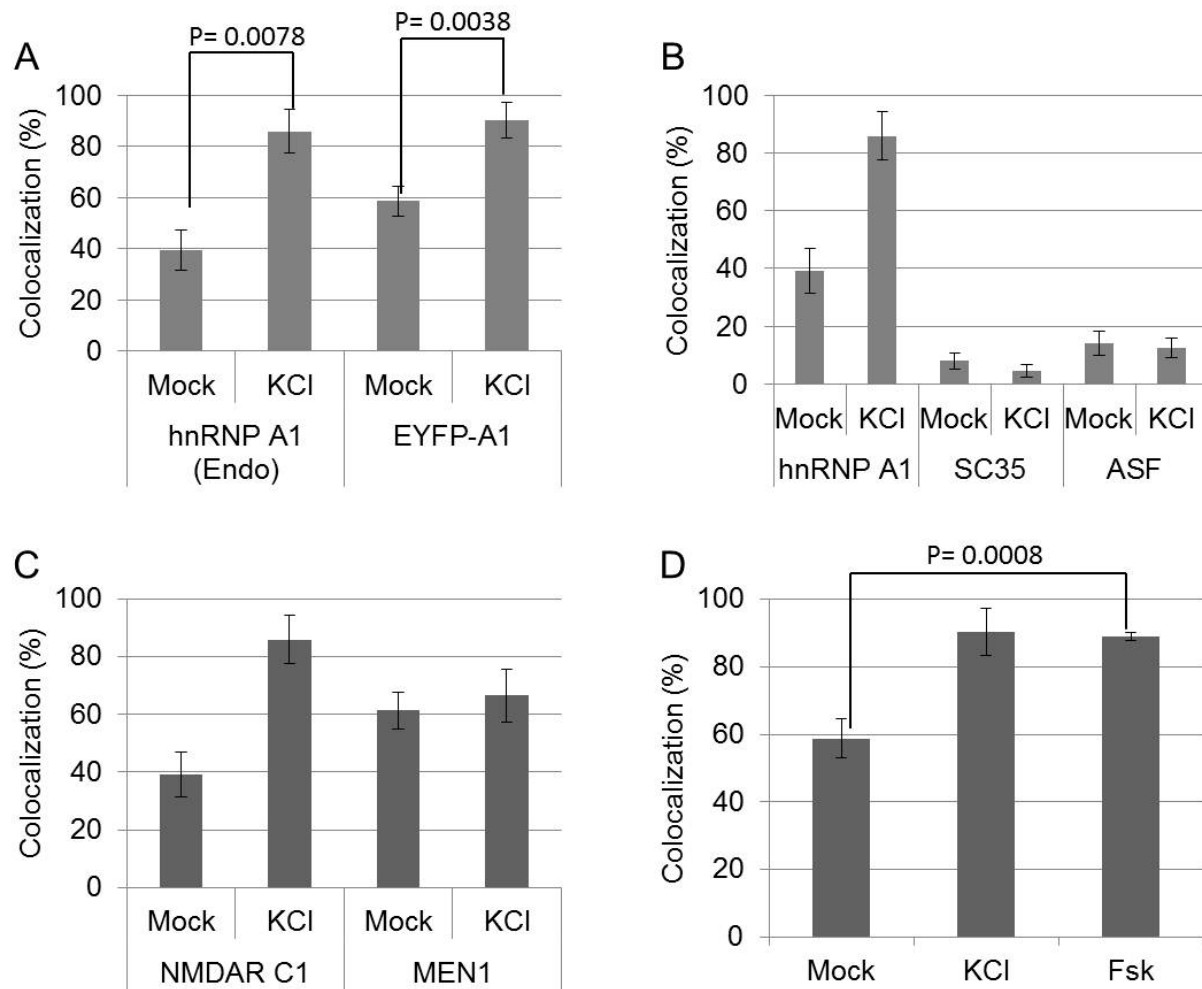


Figure 18. Quantitation results of recruitment assay

Quantitation of percentage of cells with colocalization of C1 RNA-FISH and splicing factor signals. Values represent averages from at least 80 transcription sites from at least 3 experiments \pm STDEV. (A) Both endogenous (left) and recombinant hnRNP A1 (EYFP-A, right) were recruited to C1 splicing sites upon KCl induced cell depolarization. Neuron cells were mock treated or treated with 50 mM KCl to induced cell depolarization. Cells were harvested for recruitment assay after 8 hours of treatment. (B) hnRNP A1 is recruited to C1 splicing sites, but not SC35 or ASF. (C) hnRNP A1 recruitment to C1 was induced by KCl treatment. hnRNP A1 recruitment to MEN1 was not induced by KCl treatment. (D) Treatment of cells with forsklin induced hnRNP A1 recruitment. For the forsklin treatment, neuron cells were treated with 3 μ M forsklin for 8 hours before being harvested for recruitment assay.

3.4.3 hnRNP A1 Recruitment is Time dependent and Reversible

To study the time scale of hnRNP A1 recruitment, I performed recruitment assay at different time points after depolarization treatment. To perform this assay, cells were treated with 50mM KCl for 0h (mock), 0.5h, 2h, 8h and 18h, and then fixed for the recruitment assay. As shown in Figure 19A, hnRNP A1 recruitment was increased from 39% to 61% 30 min after KCl treatment and peak recruitment was observed after 8 hours of KCl treatment in the time scale I performed. Interestingly, in the corresponding splicing assays I performed (data not shown), I did not observe significant C1 inclusion decrease until 8 hours after KCl treatment. This suggests that hnRNP A1 was recruited to C1 exon prior to the C1 exon skipping after the cell depolarization

Previous report from our lab showed the KCl induced C1 exon skipping could be reversed after KCl washout (An and Grabowski 2007). To test if the recruitment was also reversible, cells were treated with KCl for 8 hours, and then washed out and incubated in fresh media without KCl for 2 hours or 16 hours. After 2 hours of KCl removal, I observed significant decrease of hnRNP A1 recruitment (from 86% to 54%). After 16 hours of KCl removal, hnRNP A1 recruitment decreased to the basal level (Figure 19B). Interestingly, the decrease of A1 recruitment also happened prior to the C1 exon inclusion increase after the KCl removal. These results suggest that the recruitment of hnRNP A1 to C1 splicing is dynamic and reversible.

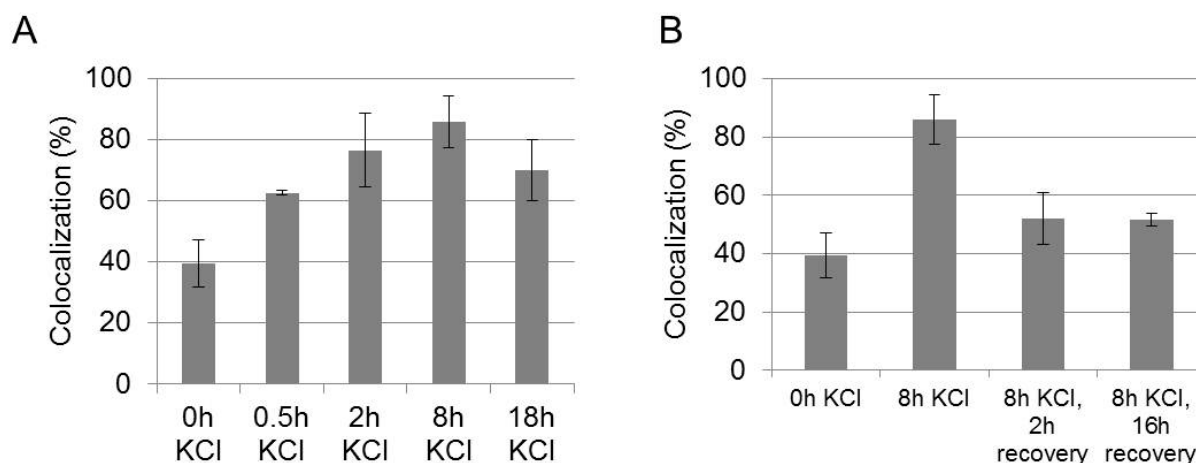


Figure 19. Recruitment of hnRNP A1 is time dependent and reversible

(A) Time lapse recruitment assay for hnRNP A1. Primary neurons at DIV8 were treated with 50 mM KCl up to 18 hours as indicated in the figure. After treatment, cells were fixed for the recruitment assay. (B) A1 recruitment is reversible after KCl washout. Primary neurons were first treated with 50 mM KCl for 8 hours, and then cultured in fresh media (no KCl) for 2 hours or 16 hours, followed by a recruitment assay.

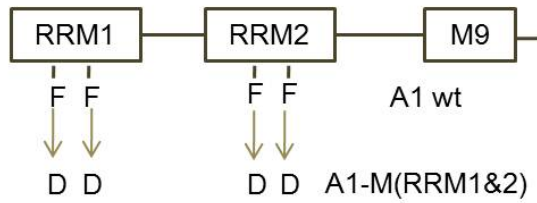
3.4.4 RNA Binding Activity is Required for hnRNP A1 Recruitment

I have shown that the hnRNP A1 is preferentially recruited to C1 exon after KCl induced cell depolarization. I then ask whether hnRNP A1 recruitment requires direct RNA contact. hnRNP A1 contains two RRM (RNA binding motif) domains at the N-terminus, which are responsible for RNA binding (Mayeda, Munroe et al. 1994). In each RRM domain, there are two amino acids, F57 F59 in RRM1 and F148 F150 in RRM2, critical for RNA binding. Mutation of these Phe to Asp (A1-M(RRM1&2), gift from Dr. Mayeda) abolishes the hnRNP A1 RNA binding ability (Mayeda, Munroe et al. 1994). Taking the advantage of this mutant, I asked whether A1-

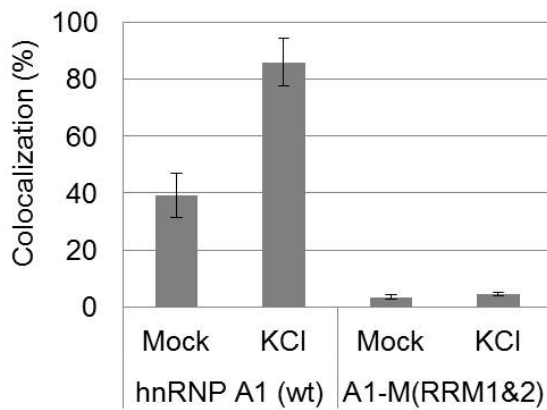
M(RRM1&2) can be recruited to C1 splicing sites in cells. To determine recruitment, A1-M(RRM1&2) was fused with EYFP at the N-terminus and transfected into primary neurons. As comparison, EYFP-A1 (wt) was used as a control for the recruitment assay. As expected, EYFP-A1-M(RRM1&2) was not recruited under either resting or depolarization conditions (Figure 20B). This suggests that hnRNP A1 recruitment to C1 splicing sites might require direct RNA binding. In addition, splicing assays using C1 splicing reporter in C2C12 cells showed that A1-M(RRM1&2) was unable to silence C1 splicing (Figure 20C). This is consistent with the recruitment assay, indicating that RNA binding is critical for both the recruitment and silencing effects of hnRNP A1.

To determine the contribution of each domains of hnRNP A1 to the splicing effect on C1 and recruitment, I constructed EYFP fusion proteins with hnRNP A1 truncation mutants (Figure 21A). Transfection of C1 splicing reporter with hnRNP A1 and truncated hnRNP A1 showed that all domains of hnRNP A1 were required for hnRNP A1 to efficiently repress C1 splicing (Figure 21B). Consistent with this, none of the truncated hnRNP A1 showed significant recruitment under either resting or depolarized conditions (Figure 21C).

A



B



C

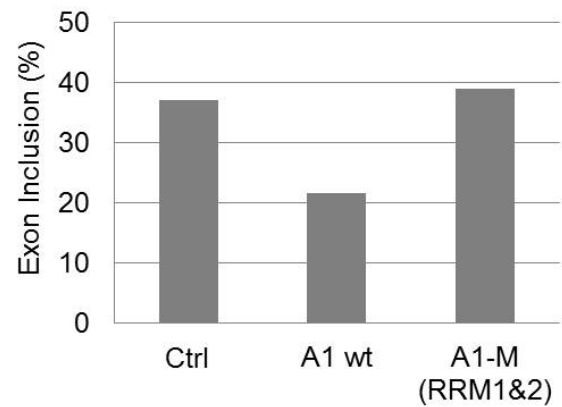


Figure 20. RNA binding is required for both hnRNP A1 recruitment and C1 splicing silencing

(A) Schematic structure of A1 wild type and A1-M(RRM1&2) mutant. Amino acids F57, F59, F148, F150 are mutated to D in the A1-M(RRM1&2) mutant. (B) RNA binding is required for A1 recruitment in vivo. Primary neurons were transfected with EYFP-A1 or EYFP-A1-M(RRM1&2) at DIV7. 18 hours after transfection, cells were either mock treated or treated with 50 mM KCl for 8 hours. Cells were then fixed and used for RNA FISH. (C) RNA binding is required for hnRNP A1 induced C1 splicing silencing. Wild type A1 or A1-M (RRM1&2) was transfected with C1 splicing reporter into C2C12 cells for 24 hours. Cells were then harvested and RNAs were isolated for splicing assay.

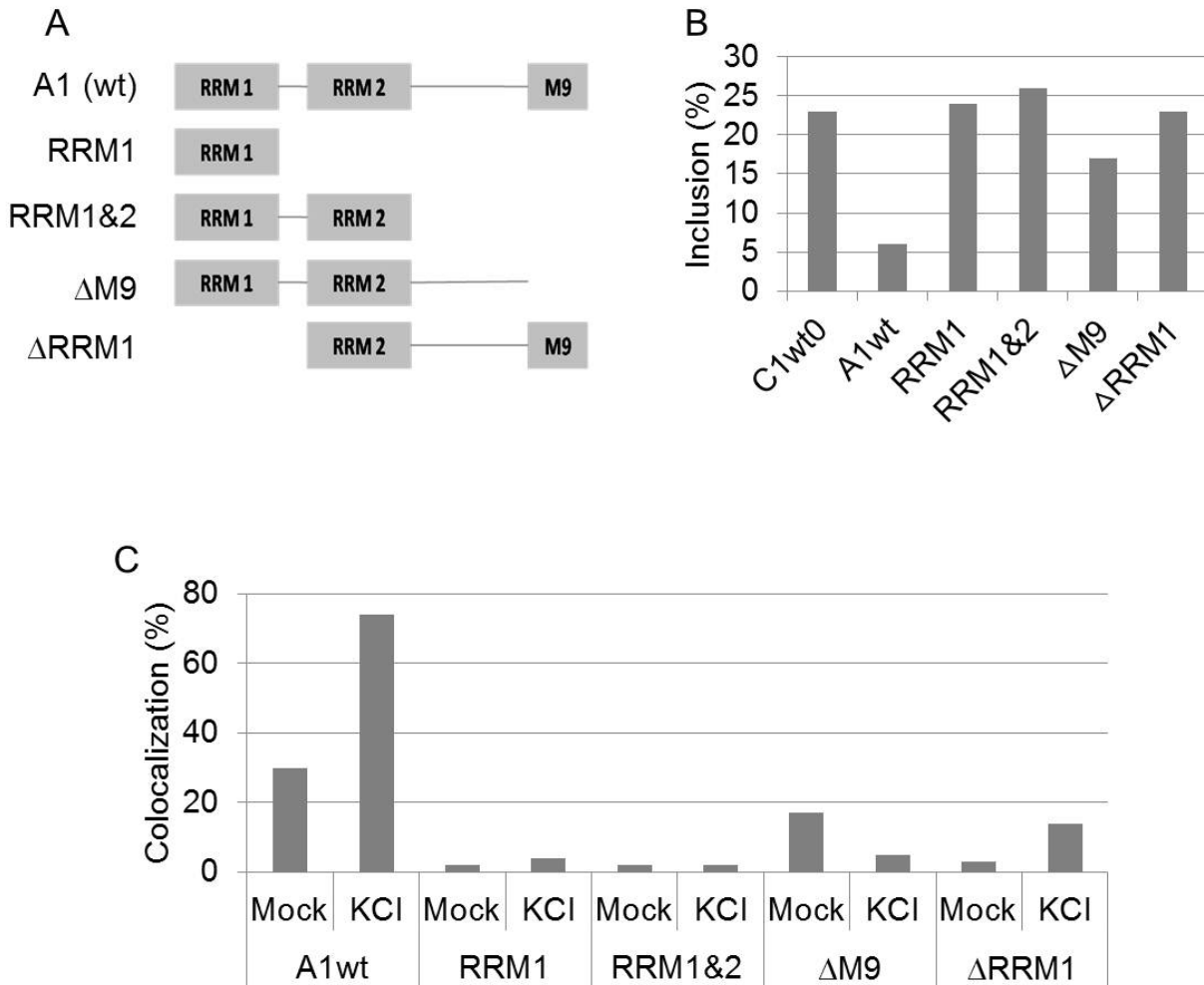


Figure 21. All domains (RRM1, RRM2 and M9) are required for hnRNP A1 induced C1 splicing silencing and hnRNP A1 recruitment

(A) Schematic structure of hnRNP A1 wild type and truncated version. (B) All domains of hnRNP A1 are required for hnRNP A1 induced C1 splicing silencing. C1 splicing reporter (or C1wt0) was transfected with A1 wild type or truncated A1 as indicated in the figure into C2C12 cells for 24 hours before being harvested for RNA isolation and splicing assay. (C) All domains of hnRNP A1 are required for hnRNP A1 recruitment to C1 splicing sites. EYFP fused A1 wild type or truncated A1 as indicated in the figure were transfected in to neuron cells for 17 hours, followed by mock or KCl treatment for another 8 hours. Cells were then harvested for a recruitment assay.

3.5 DISCUSSION

In this study, I have identified several molecular links mediating depolarization induced C1 splicing skipping. Specifically, I have shown the roles of Ca^{2+} signaling, PKA pathway and hnRNP A1 in mediating KCl induced C1 splicing silencing. Using Ca^{2+} imaging, I have demonstrated that sustained calcium influx is directly associated with depolarization induced C1 splicing silencing. Inhibition of calcium influx blocked KCl induced C1 splicing skipping; while activation of recombinant NMDA receptor in N18TG2 cells induced C1 splicing skipping. In addition, I have identified two pathways, CaMK IV and PKA pathways, which regulate C1 splicing skipping in a hnRNP A1 dependent manner. Knockdown of hnRNP A1 in N18TG2 cells or mutation of UAGG motif attenuated CaMK IV and PKA induced C1 splicing skipping. Moreover, I have also shown that hnRNP A1 was recruited to C1 splicing sites in neurons during depolarization. Taken together, I have demonstrated the roles of calcium, CaMK IV, PKA pathways and hnRNP A1 in mediating depolarization induced C1 splicing skipping. This study will provide a framework to study the signal transduction between depolarization and alternative splicing.

4.0 PKA-HNRNP A1-UAGG SILENCING MODULE

In the previous chapter, I have shown that PKA regulates C1 splicing via splicing factor hnRNP A1 and UAGG motif in the C1 exon. To further investigate whether this PKA-hnRNP A1-UAGG silencing module is transferable, I tested the silencing module in various contexts here.

4.1 PKA-UAGG SILENCING MOTIF IS TRANSFERABLE TO SIRT1A

To test whether PKA-hnRNP A1-UAGG silencing motif is transferable, I took advantage of the Sirt1a and Ess19a splicing reporters (Figure 22A). Sirt1a contains no UAGG motif and its splicing is not regulated by hnRNP A1 or PKA (Figure 22B). Ess19a was constructed by inserting 2 UAGGs into the Exon of Sirt1a. As expected, the splicing of Sirt1a did not respond to the over-expression of ca PKA. However, with insertion of 2 UAGGs, Ess19a was silenced by the over-expression of ca PKA. However, with insertion of 2 UAGGs, Ess19a was silenced by the over-expression of ca PKA (Figure 22B). In addition, mutation of UAGG to AUGG completely abolished the PKA induced splicing silencing, further confirming that the PKA induced skipping of Ess19a exon was due to the insertion of UAGG. These results suggest that UAGG motif is sufficient for PKA induced C1 splicing silencing in Ess19a.

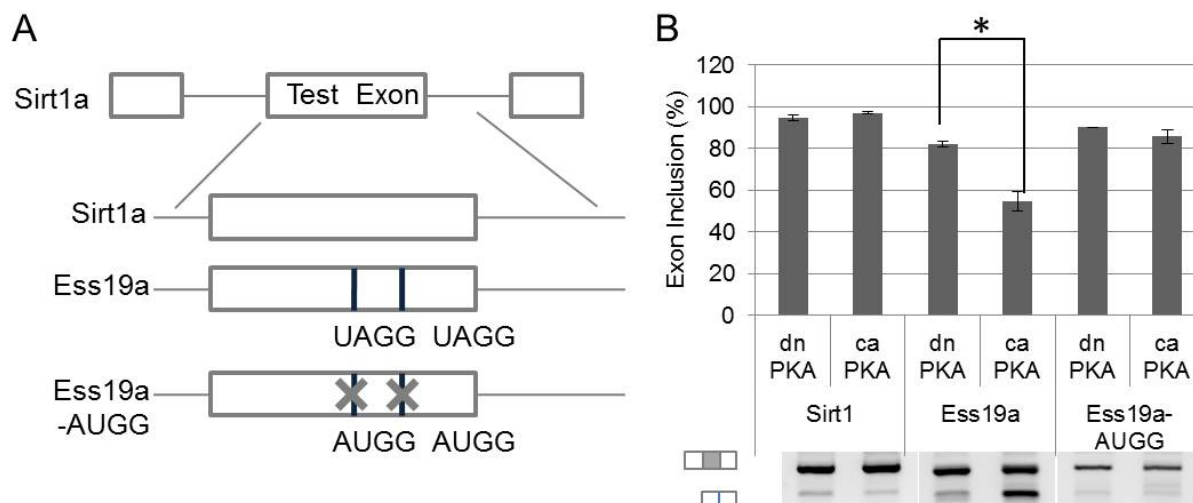


Figure 22. PKA-hnRNP A1-UAGG silencing module is transferable to Sirt1a

(A) Schematic structure of Sirt1a and Ess19a. Sirt1a contains no UAGG. Ess19a was made from Sirt1a by inserting 2 UAGGs into the exon. Relative position of UAGGs is indicated in the figure. Ess19a-AUGG was made by mutating 2 UAGGs of Ess19a into AUGG. (B) Two UAGGs in Ess19a is sufficient for PKA to induce splicing silencing. Splicing reporter Sirt1a, Ess19a and Ess19a-AUGG were transfected with dn PKA or ca PKA into N18TG2 cells for 24 hours. Transfected cells were harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.0001$).

4.2 PKA INDUCED DIP3B93WT SILENCING REQUIRES G RICH MOTIF

Alternative splicing is mediated by numerous RNA elements in the pre-mRNA. Generally, these RNA elements can be divided into two groups, enhancers and silencers, which are recognized

and bound by serine/arginine-rich (SR) and heterogeneous nuclear ribonucleoproteins (hnRNP) proteins respectively. Exon Sirt1a is a relative weakly spliced exon, with only 5 SR protein binding sites. To test if PKA-hnRNP A1-UAGG motif is also transferable to a strongly spliced exon with more SR proteins binding sites, I used the Dip splicing reporter, which contains 10 SR protein binding sites and is strongly spliced. As shown in Figure 23A, Dip3b was generated from Dip by inserting 3 UAGG motifs into the test exon. As expected, over-expression of hnRNP A1 resulted in exon skipping. Unfortunately, the splicing of Dip3b was not suppressed by the over-expression of PKA (Figure 23B). This indicates that UAGG may be not sufficient to mediate PKA induced splicing skipping in the Dip exon with a lot of SR protein binding sites, and that more hnRNP binding sites may be required. So, a G-rich motif, hnRNP H binding site, was inserted into the Dip3b, creating Dip3b93wt. Surprisingly, the splicing of Dip3b93wt was not only repressed by hnRNP A1 and hnRNP H, but also repressed by PKA, indicating both UAGG motif and G-rich motif are required for PKA induced Dip3b93wt splicing silencing.

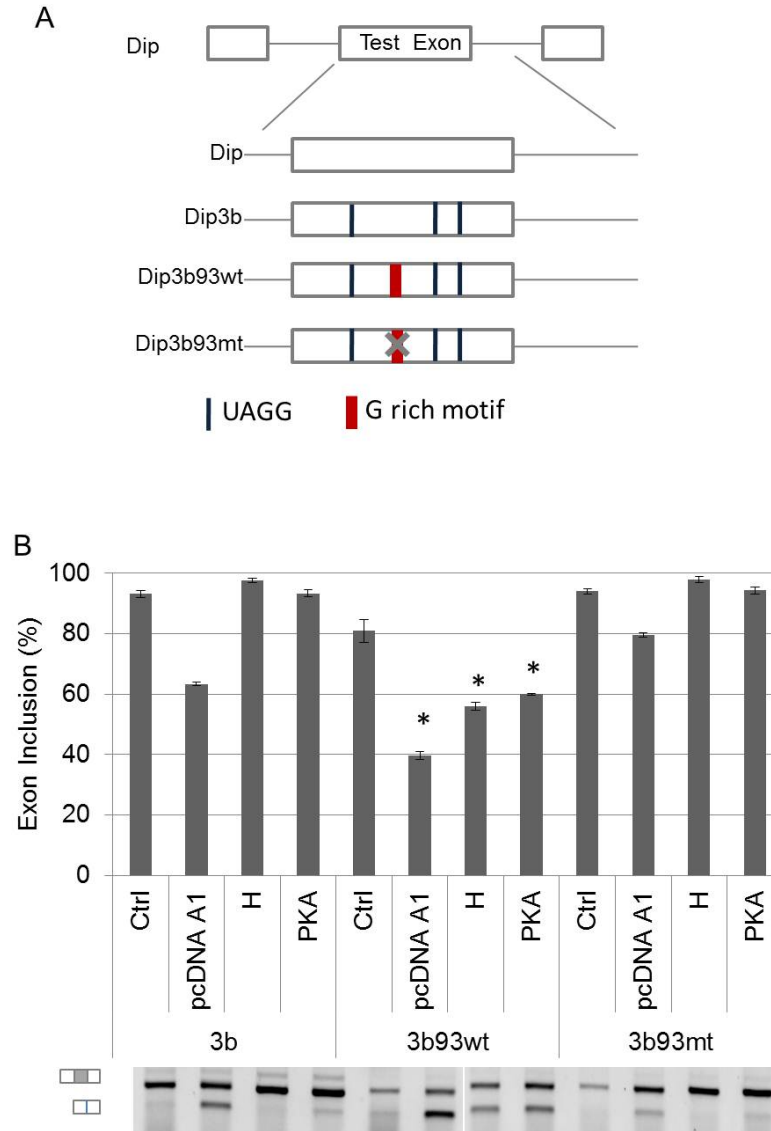


Figure 23. G-rich motif is required for PKA induced Dip splicing silencing

(A) Schematic structure of splicing reporter Dip and Dip derivatives. Relative positions of UAGG and G-rich motif are labeled with gray bar and red bar. (B) G-rich motif is required for both hnRNP H and PKA induced Dip splicing silencing. Splicing reporter Dip3b, Dip3b93wt and Dip3b93mt were transfected with vector backbone (control), A1, H and PKA into N18TG2 cells for 24 hours. Transfected cells were harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p < 0.001$).

4.3 PKA INDUCED DIP3B93WT SPLICING SILENCING IS MEDIATED BY HNRNP A1 AND HNRNP H

To test if PKA induced Dip3b93wt splicing silencing is mediated by hnRNP H and hnRNP A1, I knocked down hnRNP H and hnRNP A1 in N18TG2 cells using siRNA (Figure 24B and 25B). If PKA induced Dip3b93wt splicing silencing is hnRNP H or hnRNP A1 dependent, PKA induced Dip3b93wt splicing silencing should be attenuated in the cells in which hnRNP H or hnRNP A1 was knocked down. As expected, in the hnRNP A1 and hnRNP H knockdown cells, the PKA induced Dip3b93wt splicing silencing was attenuated about 50% (Figure 24A). To further confirm the dependence of PKA induced silencing on hnRNP A1 and hnRNP H, the binding motif of hnRNP A1 (UAGG) and hnRNP H (G-rich motif) in the exon was mutated, followed by splicing assay. As shown in Figure 23B, in Dip3b93mt, mutation of G-rich motif abolished both hnRNP H and PKA induced silencing, indicating the requirement of G-rich motif. In addition, a splicing assay with the splicing reporter Dip93, which contains G-rich motif but no UAGG motif (Figure 25C), showed that PKA induced silencing also required UAGG motif (Figure 25D). Together with the results above, these data suggest that PKA induced Dip3b93wt requires both hnRNP A1 and hnRNP H.

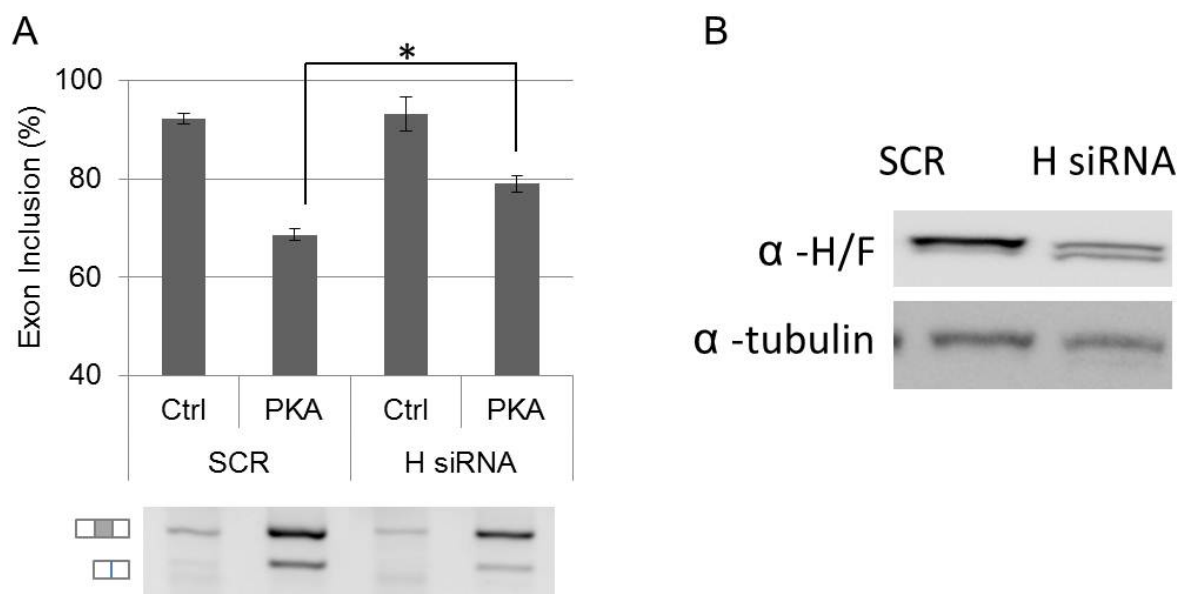


Figure 24. hnRNP H is required for PKA induced Dip splicing silencing

(A) N18TG2 cells were first treated with scrambled siRNA or hnRNP H siRNA for 24 hours, and then transfected with PKA and Dip3b93wt splicing reporter for another 24 hours. Transfected cells were harvest for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.001$). (B) N18TG2 cells were transfected with scrambled or hnRNP H siRNA for 48 hours. Transfected cells were harvested, and whole cells lysates were used for Western Blot against β -tubulin and hnRNP H as indicated in the figure. 72% hnRNP H was knocked down, as quantitated using Image Gauge 4.0.

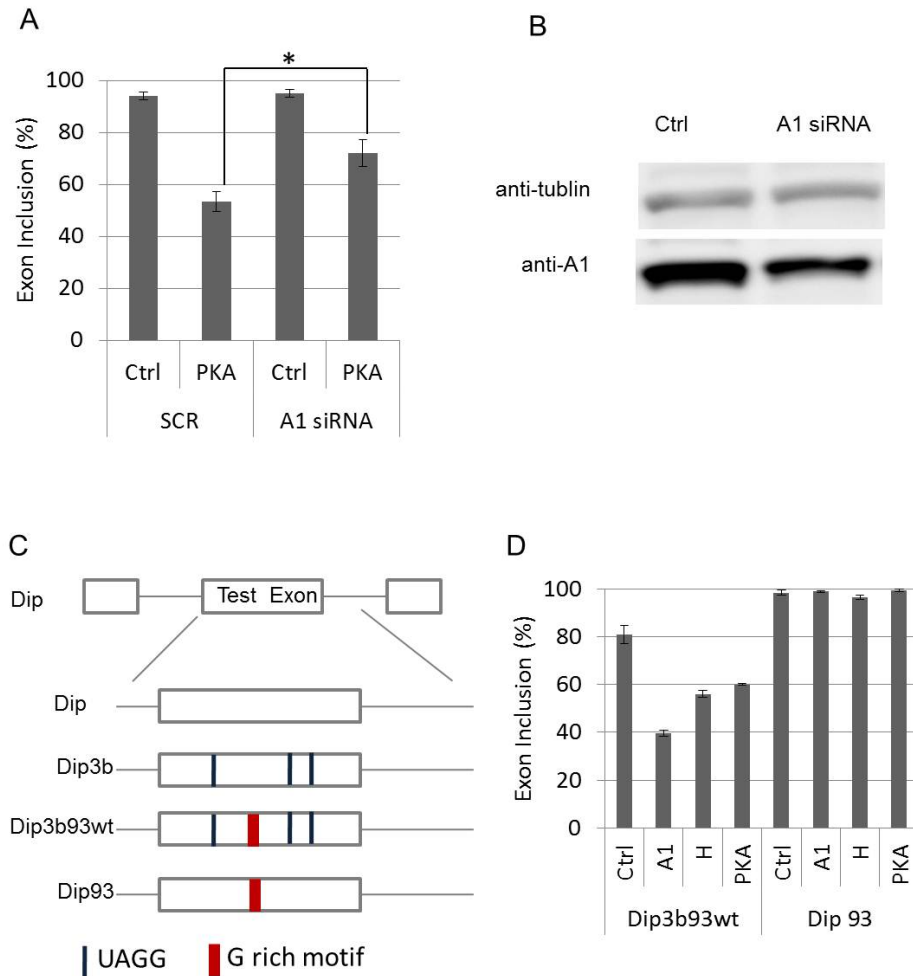


Figure 25. hnRNP A1 is required for PKA induced Dip splicing silencing

(A) N18TG2 cells were first treated with scrambled siRNA or hnRNP A1 siRNA for 24 hours, and then transfected with PKA and Dip3b93wt splicing reporter for another 24 hours. Transfected cells were harvested for RNA isolation and RT-PCR. RT-PCR products were separated on a 1.5% agarose gel ($n \geq 3$, $p=0.0071$). (B) N18TG2 cells were transfected with scrambled or hnRNP A siRNA for 48 hours. Transfected cells were harvested and whole cells lysates were used for Western blot against β -tubulin and hnRNP A as indicated in the figure. 67% hnRNP A1 was knocked down, as quantitated using Image Gauge 4.0. (C) Schematic structure of splicing reporter Dip and Dip derivatives. Relative positions of UAGG and G-rich motif are labeled with gray bar and red bar. (D) UAGG motif is required for PKA

induced Dip splicing silencing. Splicing reporter Dip3b93wt and Dip93 were transfected with vector backbone (control), hnRNP A1, hnRNP H and PKA into N18TG2 cells for a splicing assay.

4.4 HNRNP A1 AND HNRNP H REGULATE DIP3B93WT SPLICING COOPERATIVELY

Both hnRNP A1 and hnRNP H are required for PKA induced Dip3b93wt splicing silencing. This indicates that hnRNP A1 and hnRNP H might regulate Dip3b93wt splicing cooperatively. To further address this question, I first over-expressed hnRNP H with splicing reporter Dip93 and Dip3b93wt. As expected, Dip3b93wt, which contains both UAGG motif and G-rich motif, was silenced by the over-expression of hnRNP H. Surprisingly, Dip93, which contains G-rich motif but no UAGG motif, was not silenced by hnRNP H. This suggests that G-rich motif and hnRNP H may not be sufficient to induce Dip3b93wt splicing silencing and UAGG/hnRNP A1 is also required. Now the question is whether hnRNP A1 induced Dip3b93wt silencing also requires hnRNP H and G-rich motif. I over-expressed hnRNP A1 with Dip3b and Dip3b93wt. Both splicing reporters were silenced by hnRNP A1. However, the silencing effect of hnRNP A1 on Dip3b, which contains only UAGG motif, was much weaker than that on Dip3b93wt, which contains both UAGG and G-rich motif, indicating that hnRNP H might contribute to the hnRNP A1 induced Dip3b93wt splicing silencing (Figure 26B). Together with results above, these data suggest that there might be some collaboration between hnRNP A1 and hnRNP H in regulating Dip3b93wt splicing silencing.

To confirm this, I took advantage of the hnRNP A1 and hnRNP H knockdown system. Cells were first treated with hnRNP A1 or hnRNP H siRNA, and then used for splicing assay with splicing reporter Dip3b93wt. In hnRNP H knockdown cells, the hnRNP A1 silencing effect on Dip3b93wt was attenuated by 25%. In hnRNP A1 knockdown cells, the hnRNP H silencing effect on Dip3b93wt was attenuated by 50% (Figure 26C). These data further confirmed that silencing of Dip3b93wt requires activities from both hnRNP A1 and hnRNP H, suggesting that Dip3b93wt is regulated by hnRNP A1 and hnRNP H collaboratively.

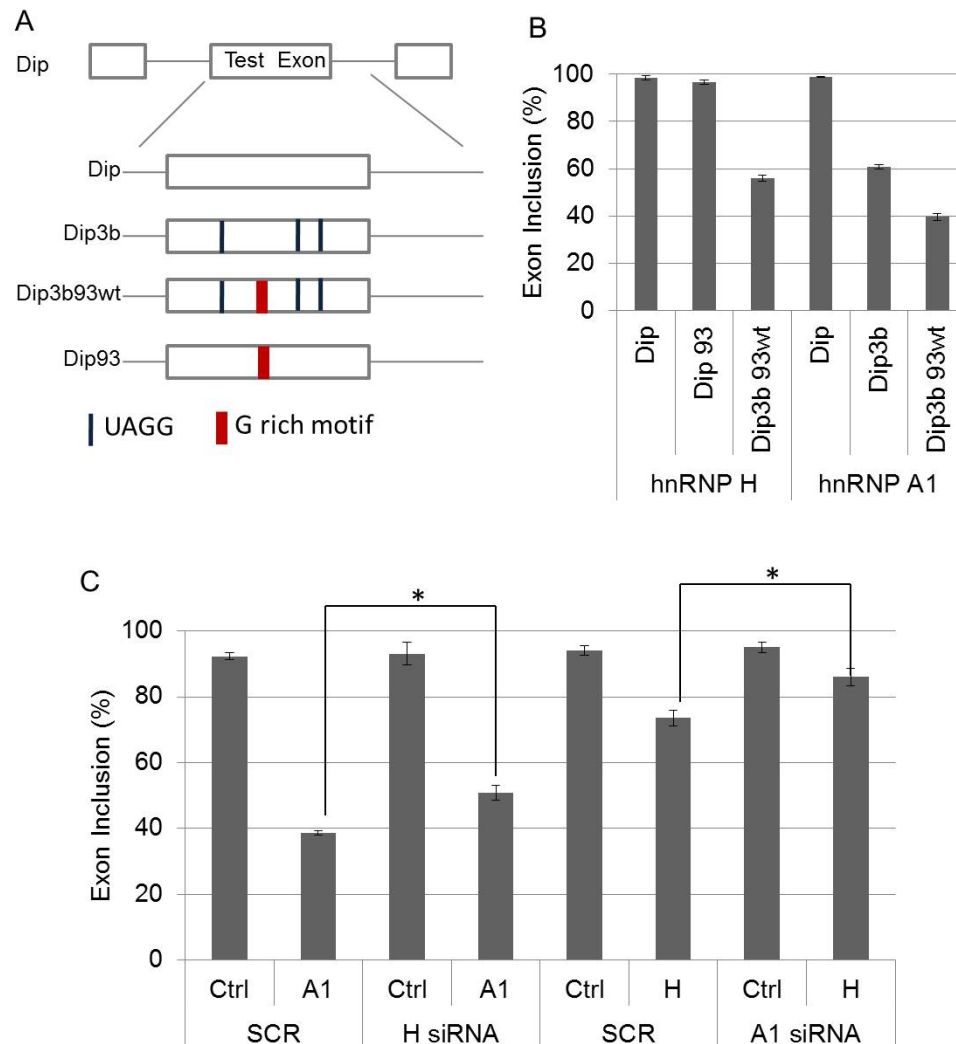


Figure 26. hnRNP A1 and hnRNP H regulate Dip3b93wt splicing collaboratively

(A) Schematic structure of splicing reporter Dip and Dip derivatives. Relative positions of UAGG and G-rich motif are labeled with gray bar and red bar. (B) hnRNP H or hnRNP A1 induced Dip3b93wt splicing silencing requires both UAGG motif and G-rich motif. Splicing reporters, as indicated in the figure, were transfected with hnRNP H or hnRNP A1 into N18TG2 cells for 24 hours. Transfected cells were then harvested for splicing assay. (C) hnRNP H or hnRNP A1 induced Dip3b93wt splicing silencing requires both hnRNP H and hnRNP A1. N18TG2 cells were transfected with scrambled or hnRNP A siRNA/hnRNP H, as indicated in the figure, for 24 hours. Cells were then transfected with splicing reporter Dip3b93wt together with hnRNP H (H) or hnRNP A1 (A1), as indicated in the figure, for another 24 hours. Transfected cells were harvested for a splicing assay ($n \geq 3$, $p < 0.01$).

4.5 DISCUSSION

In this study, I have shown that the PKA-hnRNP A1-UAGG silencing module is transferable to weakly spliced exon, with less SR protein binding sites. However, for the strongly spliced exon with more SR protein binding sites, like Dip, PKA-hnRNP A1-UAGG silencing module is not sufficient. In the case of Dip, hnRNP H silencing motif, G-rich motif, is also required. I have shown that PKA induced Dip3b93wt silencing requires both hnRNP A1 and hnRNP H activity. Moreover, I have also shown here that Dip3b93wt silencing is regulated by hnRNP A1 and hnRNP H collaboratively.

5.0 DISCUSSION AND FUTURE DIRECTIONS

In this study, I have demonstrated how the KCl induced depolarization on the cell membrane communicates with events of alternative splicing in the nucleus. Specifically, KCl induced depolarization induces sustained calcium influx, which activates CaMK IV and PKA pathways. Activation of PKA pathways will promote hnRNP A1 dimerization and increase its binding affinity to C1 exon. Recruitment of hnRNP A1 to C1 exon represses its splicing. The results from this study will contribute to define the networks that control signal-responsive alternative splicing.

5.1 KCL INDUCED C1 SPLICING SKIPPING IS MEDIATED BY SUSTAINED Ca^{2+} INFLUX

In this study, I have found that sustained Ca^{2+} influx is associated with KCl induced C1 splicing skipping. Using Ca^{2+} imaging, I monitored the calcium concentration in cells during depolarization. I found that depolarization induces a sustained Ca^{2+} influx, which is associated with C1 splicing skipping. However, treatment of cells with Ionomycin, which induces transient Ca^{2+} influx, has no effect on C1 splicing. To demonstrate that the sustained Ca^{2+} influx is

required for the KCl induced C1 splicing silencing, H89 was used to block the Ca^{2+} influx. In the cells treated with H89, both KCl induced Ca^{2+} influx and C1 splicing silencing were attenuated. In addition, treatment of MK801 and APV, which have been reported to inhibit KCl induced C1 splicing silencing (An and Grabowski 2007), also attenuated KCl induced sustained Ca^{2+} influx. Therefore, KCl induced C1 splicing silencing is associated with KCl induced sustained Ca^{2+} influx. This conclusion was further supported by the splicing assay in N18TG2 cells with the over-expression of NMDA receptors. The functionality of the NMDA receptors in N18TG2 cells was verified by Ca^{2+} imaging. Treatment of cells with NMDA receptor activators, glycine and glutamate, induced Ca^{2+} influx, indicating that the NMDA receptor subunits were functionally assembled in N18TG2 cells. Importantly, the induced Ca^{2+} influx could be blocked by NMDA receptor antagonist MK801, further confirming the functionality of the ectopic NMDA receptors. In this system, C1 splicing silencing was induced by sustained calcium influx. Importantly, the induced C1 splicing silencing could be inhibited by MK801 and APV, further confirming the role of calcium influx in regulating C1 splicing silencing.

The role of calcium in mediating C1 splicing has been implicated in a few studies (An and Grabowski 2007; Lee, Xing et al. 2007; Xie 2008). Both An and Lee have shown that C1 splicing is regulated by CaMK IV, implicating roles of Ca^{2+} in the regulation of C1 alternative splicing. However, it is not clear how the calcium concentration change in the cells will affect C1 splicing. The results from this study have shown a direct association between the Ca^{2+} concentration and C1 splicing silencing. Moreover, I have also shown that a sustained Ca^{2+} influx is required for C1 splicing skipping. This was also supported by the Ionomycin experiments. Ionomycin, which only induced transient Ca^{2+} influx, had no effect on C1 splicing in both neurons and N18TG2 cells line. Consistent with this, the NMDA receptor activity is

dynamically regulated by PKA and PP1, as shown in the Figure 29. Transient Ca^{2+} influx may not be sufficient to activate the PKA pathway and downstream alternative splicing.

5.2 KCL INDUCED C1 SPLICING IS MEDIATED BY CAMK IV AND PKA PATHWAY IN A HNRNP A1 DEPENDENT MANNER

In this study, I have shown that both CaMK IV and PKA regulate C1 splicing silencing in a hnRNP A1 dependent manner. The first evidence was from the splicing assay in hnRNP A1 knockdown cells and strengthened by the splicing assay with UAGG mutant splicing reporter. Knockdown of hnRNP A1 significantly attenuated the CaMK IV and PKA induced C1 splicing silencing, indicating the requirement for hnRNP A1. This conclusion was further strengthened by the splicing assay with UAGG mutant splicing reporter. UAGG motif in the C1 exon is critical for hnRNP A1 induced C1 splicing silencing. Mutation of UAGG motif abolished CaMK IV and PKA induced C1 splicing silencing, indicating the requirement for hnRNP A1.

CaMK IV is one of the main kinases that are directly regulated by Ca^{2+} signaling in the neuron (Ghosh and Greenberg 1995; Redmond, Kashani et al. 2002). The role of CaMK IV in regulating alternative splicing has been reported in a few studies (Xie and Black 2001; Xie, Jan et al. 2005). Although a few CaMK IV-responsive RNA elements (CaRRE) have been identified in both exons and introns, the trans-acting factors that mediate the CaMK IV and the RNA elements remain elusive. In this study, I have shown that CaMK IV regulated C1 splicing silencing is mediated by hnRNP A1. This result is consistent with and extends previous studies.

Lee and Black identified two CaRREs in the C1 exon, termed CaRRE1 and CaRRE2 (Figure 27). Mutation of either of these CaRREs abolishes CaMK IV induced C1 splicing skipping (Lee, Xing et al. 2007). Moreover, our lab has identified two UAGG motifs in the C1 exon which are critical for hnRNP A1 induced C1 splicing silencing (Han, Yeo et al. 2005; An and Grabowski 2007). Interestingly, the two CaRRE and UAGG motifs largely overlap (Figure 27). CaRRE 1 actually contains a UAGG motif at the end. Although there is no UAGG in the CaRRE 2, UAGA is found at the end of CaRRE 2. Importantly, mutation of UAGA to UAGG increased the CaMK IV silencing effect by 150% (Lee, Xing et al. 2007), indicating the UAGA might be a derivative of UAGG. The overlap between CaRRE and UAGG motifs suggests that the regulation of CaRRE and UAGG motifs might share the same pathway. In this study, I have shown that the UAGG motif, which is required for hnRNP A1 induced C1 splicing silencing, is also required for CaMK IV induced C1 splicing silencing, indicating that the UAGG motif and the CaRREs in the C1 exon are regulated by the same trans-acting factors. Knockdown of hnRNP A1 further confirmed that hnRNP A1 is the trans-acting factor that mediates CaMK IV induced C1 splicing silencing.



Figure 27. Overlap of CaRRE motif and UAGG motif in C1 exon

C1 exon contains 2 CaRRE motifs, as indicated by the box. UAGG motif and its derivative are labeled with red font.

Roles of PKA in regulating alternative splicing have been reported in a few studies. Kvissel et al. has shown that activation of PKA results in relocalization of catalytic subunits to the nucleus and phosphorylation of SR proteins (Kvissel, Orstavik et al. 2007). More recently, Li and Xie, using SELEX, have identified a CAAAAAA motif regulated by PKA (Li, Liu et al. 2009). Here, for the first time (to our knowledge), I have shown that PKA is involved in inducible alternative splicing. The first evidence was from the over-expression of ca PKA with the C1 splicing reporter. Over-expression of ca PKA reduced C1 exon inclusion by 40% (Figure 10A). Further evidence was from the knockdown and mutation experiments, which showed that PKA induced C1 splicing silencing is mediated by splicing factor hnRNP A1 and the UAGG motif in the C1 exon. Moreover, I have also shown that the PKA induced C1 splicing silencing mediated by splicing factor hnRNP A1 and UAGG motif is transferable. This indicates that the PKA-hnRNP A1-UAGG silencing module might be widely used in the regulation of alternative splicing in the absence of an abundance of ESE motifs, which antagonize the UAGG silencing effect. Note that MEN1 splicing reporter, which also contains 2 UAGG motifs in the exon, can be silenced by the over-expression of PKA in N18TG2 cells.

To investigate how hnRNP A1 is regulated, I have shown that PKA regulates hnRNP A1 splicing activity by direct phosphorylation. The first evidence was from the in vitro kinase assay with MBP-A1, showing that hnRNP A1 was phosphorylated by the PKA catalytic subunit in vitro. Although the MBP control, MBP-MS2, was also phosphorylated by PKA, the phosphorylation band was much weaker than that of MBP-A1. The phosphorylation might come from MS2, in which some phosphorylation sites are predicted by NetPhos 2.0. In addition, phosphorylation of MBP-A1 was saturated with 0.5 unit of PKA in 30 minutes. However,

saturation of MBP-MS2 phosphorylation was not reached even with 4 units of PKA in 30 minutes, indicating that MBP-MS2 is not as good a substrate as MBP-A1. Additional evidence that PKA phosphorylates hnRNP A1 is from the in vivo over-expression experiment. Jo *et al.* has developed an antibody specific for Serine 199 phosphorylated hnRNP A1 (α -S199p) (Jo, Martin et al. 2008). Although the antibody can recognize both phosphorylated and un-phosphorylated hnRNP A1, it shows preference for phosphorylated hnRNP A1 (Figure 14D). Taking advantage of this antibody, I have shown that over-expression of constitutively active PKA results in more phosphorylation of hnRNP A1. Taken together, PKA regulates hnRNP A1 activity by direct phosphorylation.

In this study, I have also shown that hnRNP A1 phosphorylation increases its RNA binding ability. The conclusion is based on the gel shift experiment showing increased RNA binding in the PKA treated hnRNP A1 samples. Consistent with this, our lab previously showed an increased binding affinity of hnRNP A1 to the C1 UAGG motif in nuclear extracts from KCl treated neurons (An and Grabowski 2007). In addition, Shi and Liu recently reported that PKA phosphorylates splicing factor ASF/SF2, and modulates its binding to tau pre-mRNA (Shi, Qian et al.), indicating it might be a common mechanism for PKA to regulate alternative splicing by phosphorylation.

Interestingly, in the native gel, I found that PKA phosphorylation promoted hnRNP A1 dimerization. This indicates that increased RNA binding affinity of hnRNP A1 might result from dimerization. This is supported by the previously published crystal structures of the N-terminus of hnRNP A1 or UP1, which contains the first two RRM domains. Without ssDNA or RNA, UP1 was crystallized as a monomer (Shamoo, Krueger et al. 1997; Xu, Jokhan et al. 1997). However, when UP1 was crystallized in complex with ssDNA d(TTAGGG)₂, it formed a dimer.

More importantly, in the complex crystal structure, DNA binding was achieved through a dimer of UP1 (Ding, Hayashi et al. 1999). Those results suggest that hnRNP A1 might function as dimer when bound to ssDNA or RNA. Consistent with this, Blanchette and Chabot presented a looping out splicing model for hnRNP A1 which was mediated by the dimerization of hnRNP A1 (Blanchette and Chabot 1999; Nasim, Hutchison et al. 2002). Thus, phosphorylation promotes hnRNP A1 dimerization, resulting in RNA binding affinity increase.

The difference in the K_d of hnRNP A1 and phosphorylated hnRNP A1 for A1 winner is relatively small (about 1.5-2 fold). A1 winner was identified by SELEX after 8 rounds of amplification and selection. The affinity of hnRNP A1 for A1 winner is 70 times higher than that for 5' or 3' splice site from the human β globin gene, and 300 times higher than that for human β globin intron (Burd and Dreyfuss 1994). Considering the extreme high binding affinity of A1 winner to hnRNP A1, the RNA binding affinity difference between hnRNP A1 and phosphorylated hnRNP A1 may be hard to be differentiated with A1 winner. In addition, the UAGG motifs in the C1 exon do not perfectly match with the A1 winner sequence. Therefore, there might be more difference between phosphorylated and un- phosphorylated hnRNP A1 on the binding of C1 full length exon in vivo. In fact, in the previously published UV-crosslinking experiment, it was shown that hnRNP A1 from depolarized neuron cells has a higher affinity for full-length C1 exon than that from untreated neuronal cells (An and Grabowski 2007).

5.3 HNRNP A1 IS SPECIFICALLY RECRUITED TO C1 SPLICING SITE UPON THE TREATMENT OF KCL

hnRNP A1 recruitment to C1 splicing sites was demonstrated by a quantitative single cell analysis. In this assay, recruitment of endogenous hnRNP A1 to the transcription sites of C1 was detected by simultaneous RNA fluorescent in situ hybridization (FISH) to visualize the sites of C1 transcription using a DNA intron probe and indirect immunofluorescence (IF) using hnRNP A1 antibodies (9H10) to detect endogenous hnRNP A1. Positive recruitment was defined by two simultaneous criteria: a) the immunofluorescence signal of hnRNP A1 accumulated at the transcription site more than 1.5-fold above average nucleoplasmic signal and b) at least half of the splicing factor intensity peak overlapped with at least half of the RNA-FISH intensity peak. Upon treatment with KCl, the recruitment of hnRNP A1 to C1 splicing sites increased significantly. This result was further verified by the recruitment assay using recombinant hnRNP A1 (EYFP-A1) and DNA/LNA probe, which binds specifically to the exon/intron junction. These results suggest that hnRNP A1 was relocated to C1 splicing sites during cell depolarization. This could result from increased hnRNP A1 binding affinity by depolarization. Alternatively, according to the silencer zone model (Black 2003), depolarization could promote hnRNP A1 multimerization on the C1 exon, thus resulting in accumulation of hnRNP A1 at the C1 splicing sites.

These results are consistent with and extend our previous biochemistry results. Previously our lab showed an increased binding affinity of hnRNP A1 to the C1 UAGG motif in nuclear extract from KCl treated neurons (An and Grabowski 2007), suggesting that KCl induced depolarization might change hnRNP A1 binding affinity to C1 exon. In this study, using an in

vivo method, I have demonstrated that hnRNP A1 is recruited to C1 splicing sites upon depolarization. These in vivo and in vitro results suggest that KCl induced depolarization changes hnRNP A1 RNA binding affinity. Considering the roles of PKA in regulating C1 splicing, it is likely that depolarization activates the PKA pathway, which phosphorylates hnRNP A1 and promotes hnRNP A1 binding. This is supported by results from the recruitment assay performed in the presence of the PKA activator, forskolin (Figure 18D). Treatment of neurons with 3 μ M forskolin could mimic the hnRNP A1 recruitment induced by depolarization in the neuron cells, suggesting that the PKA pathway play a role in mediating depolarization induced C1 splicing silencing. In addition, hnRNP A1 was phosphorylated at site S199 by KCl treatment, indicating activation of the PKA pathway. Together, these results suggest that depolarization induced hnRNP A1 recruitment is mediated by the PKA pathway.

hnRNP A1 is widely studied as a splicing silencer. Recruitment of hnRNP A1 represses splicing. Gatto-Konczak and Breathnach have previously shown that tethering hnRNP A1 to exon K-SAM can repress its splicing (Del Gatto-Konczak, Olive et al. 1999). Consistent with this, in our recruitment assay, more hnRNP A1 recruitment was detected in the KCl treated samples, where more C1 splicing was repressed. hnRNP A1 contains 2 RRM domains at N-terminal, and a glycine-rich domain at C-terminal. The RRM domain and glycine-rich domain of hnRNP A1 are modular in function. It has been shown that recruitment of only the glycine-rich is sufficient to repress K-SAM exon splicing (Del Gatto-Konczak, Olive et al. 1999). This indicates that the first 2 RRM domains are responsible for RNA binding, and that mutation in the RRM domains will abolish hnRNP A1 recruitment and its silencing effect (Del Gatto-Konczak, Olive et al. 1999). Consistent with this, in this study I have shown that mutation of the critical amino

acids in RRM domains (F57 F59 in RRM1 and F148 F150 in RRM2), which are responsible for RNA binding, completely abolished its recruitment to C1 exon, and its silencing effect on C1.

5.4 MODEL OF KCL INDUCED C1 SPLICING SILENCING

From the results of this study, I have generated a model for the depolarization induced C1 exon splicing silencing (Figure 30). First, depolarization on the cell membrane induces Ca^{2+} influx resulting in an increase of intracellular Ca^{2+} concentration and activation of CaMK IV and PKA. CaMK IV and PKA are two of main kinases that are directly activated by Ca^{2+} signaling in neurons. The roles of CaMK IV in mediating KCl induced C1 splicing have been demonstrated in this study as well as in others (An and Grabowski 2007; Lee, Xing et al. 2007). The roles of PKA in mediating KCl induced C1 splicing silencing is supported by 1) over-expression of ca PKA in N18TG2 cells results in C1 splicing silencing, 2) activation of PKA pathway can mimic the KCl induced hnRNP A1 recruitment in neurons and 3) KCl treatment results in phosphorylation of hnRNP A1 at S199 in neurons. In addition, the relationship between the CaMK IV and PKA pathway has been investigated by dominant negative PKA saturation experiment, which indicates that CaMK IV might work upstream of PKA in regulating C1 splicing silencing. However, the mechanism by which PKA is regulated by CaMK IV is not clear.

Secondly, activation of PKA pathway phosphorylates hnRNP A1 and increases its RNA binding affinity, as supported by in vivo and in vitro phosphorylation and gel shift experiments. Interestingly, phosphorylation of hnRNP A1 can also promote hnRNP A1 dimerization. Although the direct evidence between hnRNP A1 dimerization and its RNA binding affinity is

missing, both crystal structure results and previously proposed hnRNP A1 silencing model support the view that hnRNP A1 may bind to RNA as a dimer (Blanchette and Chabot 1999; Ding, Hayashi et al. 1999). Thus, the increased hnRNP A1 RNA binding affinity might result from the dimerization of hnRNP A1, which may be promoted by phosphorylation. Finally, recruitment of hnRNP A1 to C1 exon results in C1 exon splicing silencing.

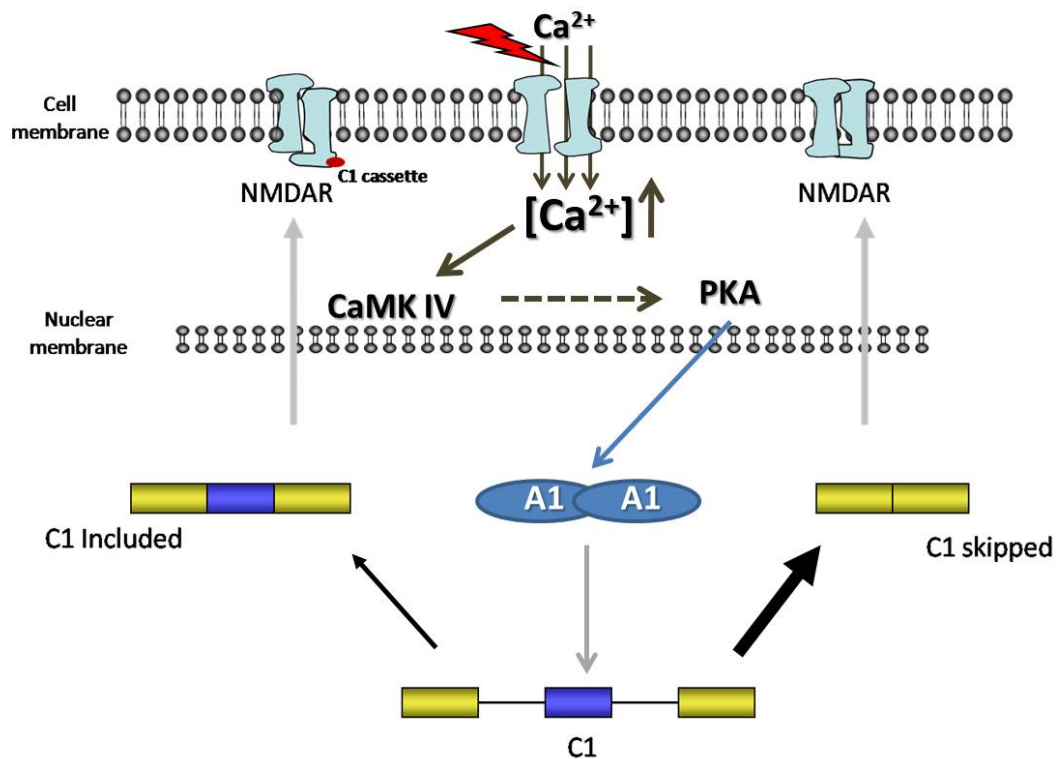


Figure 28. Model of KCl induced C1 splicing silencing

Depolarization on the cell membrane induces Ca^{2+} influx, which results in increase of cellular Ca^{2+} concentration. Ca^{2+} concentration increase activates the CaMK IV and PKA pathway. Activation of PKA results in hnRNP A1 phosphorylation and promotes hnRNP A1 dimerization. Phosphorylation of hnRNP A1 increases its binding affinity for C1 exon, resulting in hnRNP A1 recruitment. Recruitment of hnRNP A1 to C1 exon represses its splicing.

5.5 C1 SPLICING AND SYNAPTIC HOMEOSTASIS

The synaptic homeostasis model holds that, in order to stabilize excitability, neurons tend to adjust their synaptic strengths in response to external stimuli (Burrone and Murthy 2003). This model, together with the results shown here, suggest that alternative splicing of the C1 cassette exon might be involved in strategies in response to antagonize the external stimuli—KCl treatment. Interestingly, the PKA pathway is also involved in this process. It has been reported that Ca^{2+} influx activates the PKA pathway (Grewal, Horgan et al. 2000). In turn, activation of the PKA pathway is required to augment the Ca^{2+} influx via NMDA receptor (Skeberdis, Chevaleyre et al. 2006). Consistent with this, this study shows that PKA activity is required for the Ca^{2+} influx in neurons. Treatment of cells with a PKA inhibitor, H89, resulted in attenuation of KCl induced Ca^{2+} influx, and pre-incubation of cells with H89 resulted in no influx. These results indicate the critical roles of PKA in regulating Ca^{2+} influx via NMDA receptors.

Meanwhile, I have also shown that PKA regulates C1 splicing by repressing its splicing via splicing factor hnRNP A1. Interestingly, the C1 cassette in the NR1 subunit of NMDA receptor is critical for PKA activity in augmenting Ca^{2+} influx. PKA has been reported to be associated with NMDA receptor via yotiao protein (Westphal, Tavalin et al. 1999), which interacts with NMDA receptor in a C1 cassette dependent manner (Figure 29). Therefore, the activation of PKA promotes C1 splicing skipping and increases the pool of NR1 subunits without C1 cassette, which in turn will abolish PKA activity and attenuate Ca^{2+} influx through NMDA receptor. Thus, synaptic homeostasis is achieved by regulating the PKA activity via the C1 cassette in the NMDA receptor. Interestingly, the gene encoding the scaffold protein yotiao, which mediates the association between PKA and NMDA receptor, is also alternatively spliced

(Westphal, Tavalin et al. 1999). However, it is not clear whether the splicing is regulated by PKA and is involved in synaptic homeostasis.

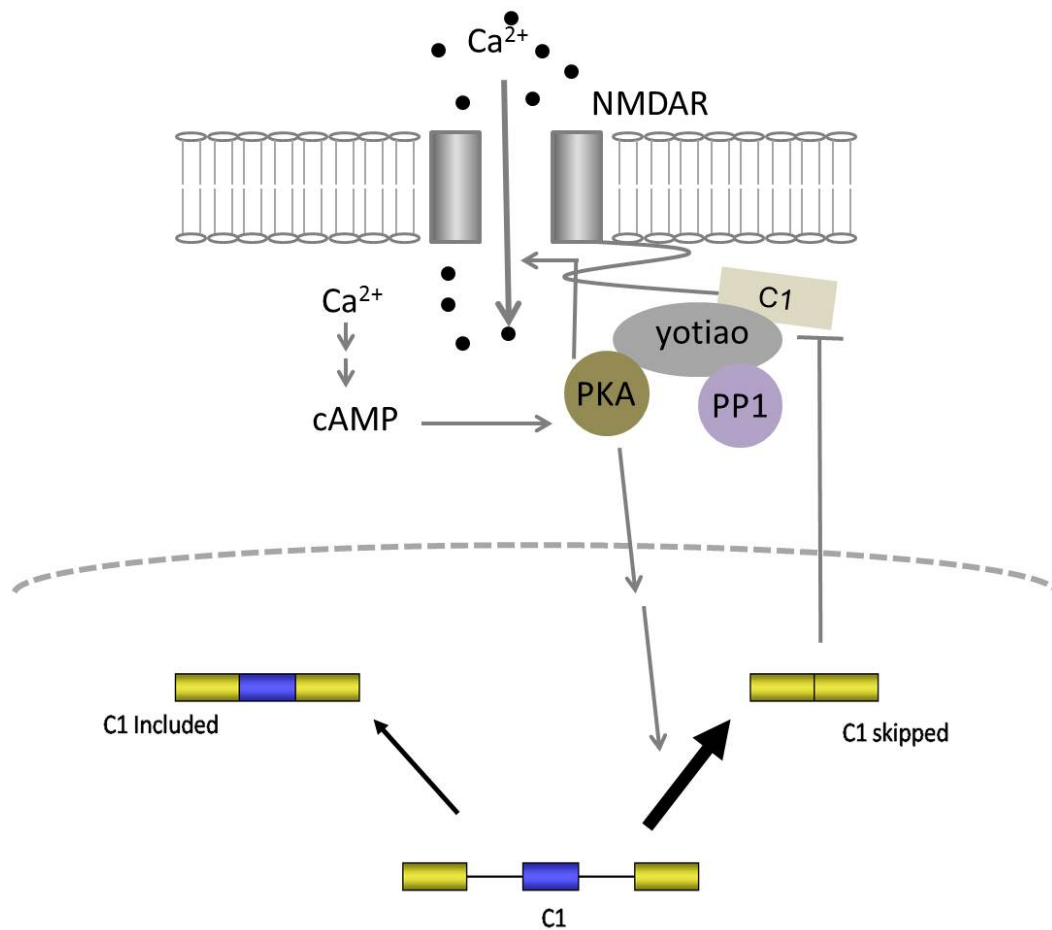


Figure 29. Model of PKA mediated synaptic homeostasis

Calcium influx through NMDAR activates PKA via cAMP pathway. Activation of PKA in turn augments NMDAR calcium influx. Meanwhile, activation of PKA also promotes C1 splicing skipping and increase the pool of NR1 subunits without C1 cassette. The association of PKA with NMDA receptor is mediated by yotiao, which is C1 cassette dependent. The lack of C1 cassette in the NMDA receptor will abolish PKA NMDA receptor complex, resulting in attenuation of PKA activated calcium influx. Thus, synaptic homeostasis is achieved.

NMDA receptor plays critical roles in excitatory synaptic transmission, plasticity and excitotoxicity in the neuronal cells. Its activity is highly regulated. Prolonged stimulation of NMDA receptor could result in neurotoxicity and disease, while missing of NMDA receptor activity in mice results in birth death (Olneyb 1987).

It has been reported that excessive NMDAR activity is the main contributor to the pathogenesis of Huntington's disease (Milnerwood, Gladding et al. 2010). Neurons expressing high levels of NMDARs are lost early from the striatum of individuals affected with Huntington's disease, and injection of NMDAR agonists into the striatum of rodents or non-human primates recapitulates the pattern of neuronal damage observed in Huntington's disease (Fan and Raymond 2007). Altered NMDAR function has been reported in corticostriatal synapses in the mouse model of Huntington's disease, and NMDAR-mediated current and/or toxicity have been found to be potentiated in striatal neurons from several HD mouse models (Fernandes and Raymond 2009). Changes in NMDAR activity have been correlated with altered calcium homeostasis, membrane depolarization and caspase activation (Fernandes and Raymond 2009). Therefore, NMDAR receptor has been shown to be a potential targets for effective and selective therapies for Huntington's disease. In addition to Huntington's disease, excessive NMDAR activity also contribute (at least partly) to acute ischaemic stroke, traumatic brain, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis and prion disease (Kalia, Kalia et al. 2008).

NMDA receptor is one of main calcium channels in neurons. Calcium is ubiquitous intracellular messengers governing a large number of cellular functions such as the control of cell growth and differentiation, membrane excitability, exocytosis, and synaptic activity (Sattler and Tymianski 2000). Although neurons have evolved complex homeostatic mechanisms to

control the calcium concentration in cells, excessive calcium loading, exceeding the capacity of Ca^{2+} -regulatory mechanisms, may inappropriately activate Ca^{2+} -dependent processes (Bindokas and Miller 1995). When overactivated, such processes including enzymes (e.g., proteases, lipases, endonucleases) and other metabolic machinery directly damage neurons or lead to the formation of toxic reaction products which ultimately cause cell death (Sattler and Tymianski 2000; Arundine and Tymianski 2003).

In this study, I have demonstrated a homeostasis model of NMDA receptor. In this model, calcium influx through NMDA receptor induces C1 exon skipping, while the missing of C1 cassette in the NR1 subunit in turn reduces the NMDA receptor activity. Thus, excessive NMDAR activity is reduced and the intracellular calcium homeostasis is achieved. NMDA receptor related diseases are mainly caused by excessive NMDAR activity. Therefore, the C1 cassette, which mediates the intracellular calcium homeostasis, might provide a potential target for new therapeutic approaches to these disease.

5.6 PKA-HNRNP A1-UAGG SILENCING MODULE

I have shown here that PKA-hnRNP A1-UAGG silencing module is transferable. In the case of the Sirt1a splicing reporter, insertion of 2 UAGGs is sufficient to be silenced by PKA and hnRNP A1. This was further confirmed by the Ess19a-AUGG mutant. In the case of Dip splicing reporter, however, an additional G-rich motif is required for PKA induced silencing. This result was confirmed by mutagenesis, which showed that both the UAGG and G-rich motifs were required for PKA induced splicing silencing. Further support was from the hnRNP A1 and

hnRNP H knockdown experiments, which showed that both hnRNP A1 and hnRNP H were required for PKA induced Dip splicing silencing. In addition, I have shown that hnRNP A1 and hnRNP H regulate Dip3b93wt collaboratively. This was supported by the UAGG and G-rich motif mutation experiment. Mutation of UAGG not only affected the hnRNP A1 silencing effect, but also abolished the hnRNP H silencing effect. And the mutation of G-rich motif also attenuated hnRNP A1 induced silencing. Additional support was from the hnRNP A1 and hnRNP H knockdown experiment. Knockdown of hnRNP A1 or hnRNP H would affect each other's silencing effect on Dip3b93wt.

The results here are consistent with and extend previous studies. UAGG silencing motif was first identified in the C1 exon, and the silencing was mediated by splicing factor hnRNP A1 (Han, Yeo et al. 2005). However, it was not clear how hnRNP A1 was regulated. In this study, I have shown that hnRNP A1 activity is regulated by PKA. Therefore, I have extended the hnRNP A1-UAGG silencing motif to PKA-hnRNP A1-UAGG silencing module. I have tested this silencing module in Sirt1a and Dip splicing reporter. In Sirt1a, this PKA-hnRNP A1-UAGG silencing module is transferable. However, in the case Dip, additional silencing motif, G-rich motif, is required. This might result from the additional SR binding sites in the Dip splicing reporter. Alternative splicing is mediated by numerous RNA elements in the pre-mRNA, including enhancers and silencers. Observed splicing patterns are often a result of the dynamic balance of enhancing and silencing functions. In the exon of Sirt1a, there are only 5 SR protein binding sites, while more than 10 SR protein binding sites are found in the exon of Dip, as predicted by ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?_process=home). This suggests that Dip might be harder to be silenced than Sirt1a. This was supported by the splicing assay. In Sirt1a, insertion of two UAGGs is sufficient to be silenced by hnRNP A1,

while Dip needed 3 UAGGs. Over-expression of hnRNP A1 with Dip2b splicing reporter (with 2 UAGG insertions) resulted in minimal silencing of Dip2b (data not shown). Therefore, in the case of Dip, an additional silencing motif may be required for PKA induced silencing.

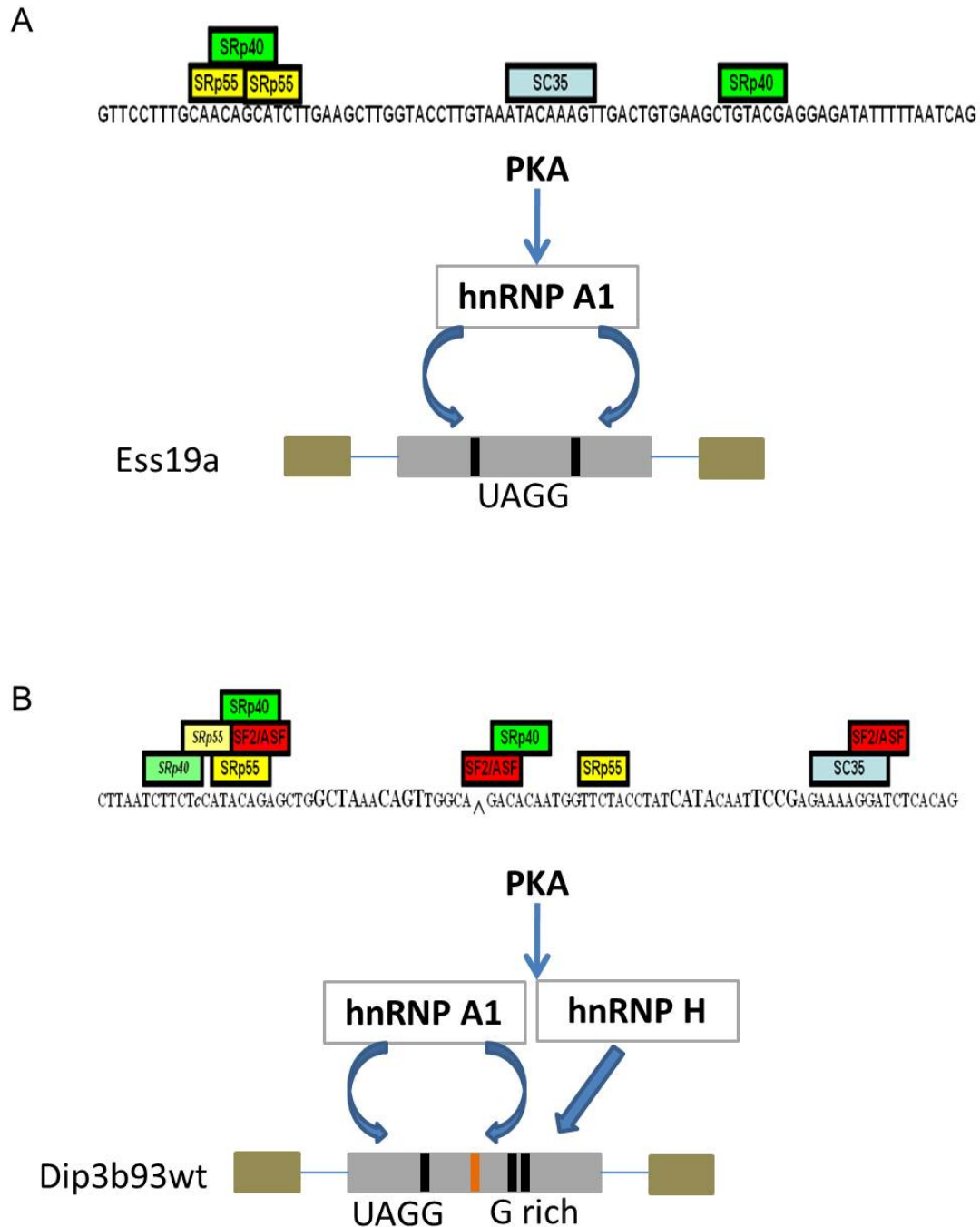


Figure 30. Model of PKA induced splicing silencing

(A) In the case of weakly spliced exon, such as splicing reporter Sirt1a, which contains few SR protein binding sites, UAGG motif is sufficient for PKA to induce splicing silencing. PKA activates hnRNP A1 by phosphorylation. Activated hnRNP A1 binds to the UAGG in the exon and represses splicing.

Sequence of Ess19a and SR protein binding sites are shown in the figure. Relative positions of UAGG in the exon are labeled with black bar. (B) In the case of strongly spliced exon, such as splicing reporter Dip, which contains many SR protein binding sites, G-rich motif and hnRNP H are required for PKA induced splicing silencing. PKA activated hnRNP A1 and hnRNP H might work cooperatively to repress the exon splicing. Sequence of Dip3b93wt and its SR protein binding sites are shown in the figure. Relative positions of UAGG and G-rich motif in the exon are labeled with black bar and red bar.

5.7 FUTURE DIRECTIONS

Although this study has answered many questions about the communication between the depolarization and splicing decision, it has raised even more questions. In this study, I have indicated that CaMK IV might work upstream of the PKA pathway. However, the mechanism by which PKA is regulated by CaMK IV is not clear. In addition, considering the abundance of CaMK IV in the nucleus, is it possible that CaMK IV might regulate hnRNP A1 directly by phosphorylation? An in vitro and in vivo kinase assay with CaMK IV should be able to answer this question.

I have shown that PKA phosphorylates hnRNP A1 and increases its RNA binding affinity to the A1 winner. It will be interesting to investigate if in vitro phosphorylation of hnRNP A1 changes its splicing activity. One way to answer this question would be to use a in vitro splicing assay. In vivo hnRNP A1 may need to be depleted. Recombinant hnRNP A1 with or without treatment of PKA will be used for the assay. This assay will provide direct evidence about the

effect of phosphorylation on hnRNP A1 splicing activity. I have also shown that KCl induces hnRNP A1 recruitment. Therefore, it will be interesting to investigate whether tethering of hnRNP A1 will repress C1 splicing, and whether tethering G-rich domain is sufficient to repress C1 splicing.

In this study, I have also shown that PKA regulates hnRNP A1 activity by direct phosphorylation. How many splicing factors are regulated by PKA? Does PKA regulate other splicing factor by direct phosphorylation? In the case of Dip3b93wt, PKA induced splicing silencing requires the activity from hnRNP H. So the question is whether PKA regulates the activity of hnRNP H by direct phosphorylation? If so, where is the phosphorylation site and how phosphorylation can change the activity of hnRNP H?

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